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Contents

Original papers

Reviews

Evaluation of the three methods of bacterial decontamination on implants with three different surfaces

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A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation; D – writing the article; E – critical revision of the article; F – final approval of the article

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Abstract

Background. The main goal of the treatment of the peri-implantitis is to decontaminate the surface of the implant, thereby enabling further treatment involving, e.g., guided bone regeneration. Since new implants of the rougher surface were introduced to the common dental practice, decontamination is even more difficult.

Objectives. The aim of the study was to evaluate 3 different methods of decontaminating implants with 3 different surfaces.

Material and methods. A total of 30 dental implants with 3 different surface types (machined, sandblasted, and acid-etched (SLA) and hydroxyapatite (HA)-coated) were used in the study. Each group of implants was coated with *Escherichia coli* biofilm and cultivated. Afterwards, the implants were transferred to the jaw model and treated with a different method: sonic scaler mechanical debridement with a Woodpecker PT5 sonic scaler (1st group), and mechanical debridement with sonic scaler and with the combination with chemical agent Perisolv® (2nd group), and with Er:YAG laser treatment (3rd group). Each implant was treated with the specific method and sent for further microbiological evaluation.

Results. The highest level of decontamination was achieved for machined-surface implants and the lowest for HA-coated implants. The method with the highest biofilm reduction was the scaler and Perisolv® group. The highest level of decontamination of HA-coated implants were achieved for Er:YAG laser irradiation method.

Conclusions. In the following paper, the superiority of combined chemical-mechanical method of decontaminating the surface of the implant on SLA and machined-surface implants was proved. On the contrary, Er:YAG laser irradiation was reported as the best option for decontamination of the HA-coated implants. In our opinion, it is a significant finding, revealing that the method of peri-implantitis management should be considered in accordance to the type of the surface of the implant (customized to the surface of the implant).

Key words: implant surface, implant surface treatment, bacterial coating

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Introduction

With the increasing number of patients treated with dental implants, a corresponding number of post-treatment complications can be expected. The most common complication in dental implant therapy is peri-implantitis.[1] It is defined as an inflammatory reaction that affects the hard and soft tissue, which results in the loss of supporting bone and gingival pocket formation surrounding the functioning osseointegrated implant.[2] This pathological condition is caused by a polymicrobial aggressive biofilm that colonizes the implant and abutment surface at the peri-implant crevice level. It is reported that its prevalence can rise up to 56%.[1–3] Anaerobic Gram-negative organisms are most commonly found in peri-implantitis-affected sites and include in among others: *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, *Peptostreptococcus micros*, *Campylobacter rectus*, *Fusobacterium* spp., and *Prevotella intermedia*, although there are also studies reporting the role of enteric rods (mostly *Escherichia coli* and *Enterobacter cloace*) in this pathology, especially at its early stage.[4,5]

Because of its complexity, peri-implantitis is still challenging to treat. Treatment involves decontamination and guided bone and tissue regeneration techniques. The decontamination process is especially difficult because the method applied can destroy the fragile surface of the implant. For this purpose a number of mechanical interventions (e.g., abrasive air powder, Teflon and plastic curettes, ultrasonic devices) and chemical agents (e.g., chlorhexidine, hydrogen peroxide) solely or in combination have been described as methods for implant surface decontamination. Although all mentioned procedures result in compromise, a successful gold standard method has not been yet established. An acceptable cleaning technique must be able to debride and detoxify the surface without traumatizing it. Decontamination with a laser, photodynamic therapy (PDT) and the application of chlorhexidine (CHX) does not seem to alter the surfaces of the dental implants. However, PDT can make an adhesive layer on the surface of the treated implants, which can facilitate new plaque formation.[6,7] Recent studies have reported that lasers can also be used in peri-implantitis management. Previously, high-power CO_2, diode and erbium lasers were used frequently, due to their hemostatic properties, selective calculus ablation and bactericidal effects. However, high-power lasers can cause an undesired increase of temperature and have been recently replaced by Er:YAG laser. Another disadvantage of lasers is the high cost of equipment.[8–10]

Dental implant surface decontamination has become even more complicated since the introduction of dental implants with improved osteoconductive properties. Machined-surface implants, which have been used for decades, have been replaced by implants characterized by a rougher surface. There are 2 main paths that can be followed in order to improve the osteoconductivity of the titanium implants. These approaches can be classified into the following 2 techniques: metallic implants are coated with the bioactive compounds that accelerate bone formation or a rough surface is formed directly on the metallic implants.[11] Both techniques increase the roughness of the surface of the implant, making osteointegration more favorable. However, as a result, it facilitates biofilm formation on dental implant surfaces.[1,2,3,6] To the best of our knowledge, there are very few studies that evaluate various decontamination methods on different surfaces of the implants.

Material and methods

The study was conducted on a total number of 30 dental implants. Implants were divided into 3 equal groups with 10 implants in each group. All the implants had the same length and diameter of L12Ø4 mm. The 1st group was machined-surface (M) implants (SGS Dental Implant System Holding – Zn, St. Gallen, Switzerland). In the 2nd group, Denium Superline II (Dentium, Seoul, South Korea) sandblasted and acid-etched dental implants (SLA) were used. The 2rd group (HA) included the hydroxyapatite (HA)-coated dental implants (SGS Dental Implant System Holding – Zn).

Bacterial cultivation

Peri-implantitis is caused by Gram-negative and anaerobic bacteria and *E. coli* were used as a model for Gram-negative bacteria. The reasons we did so is that there are many studies involving bacterial adhesion and decontamination carried out on dental implants with *E. coli* as bacteria of choice, as well as because it is a readily available and easily cultivated aerobic microorganism.

Material

The McConkey's medium (BioMaxima SA, Lublin, Poland); Sugar broth (BioMaxima SA); Saponin (Sigma-Aldrich, St. Louis, USA); reference strain: *E. coli* ATCC 25922.

Conduct of the experiment

Preparation of the inoculums

The *E. coli* ATCC 25922 strain from McConkey's medium was seeded into sugar broth and incubated at 37°C for 24 h. From the obtained culture in sugar broth, an inoculum with a density of 0.5 on the McFarland Scale (MFa) was prepared.

Implants coating

The inoculum prepared in this way, in the amount of 500 µL, was inoculated with 50 mL of sugar broth. Then, the implant was aseptically inserted and the whole was incubated at 37°C for 24 h.

Preparation of implants for further tests

After this time, the implants were removed from the culture and rinsed 3 times, in each case, in 10 mL of sterile saline to remove the plankton forms of the culture, leaving only the biofilm formed by *E. coli* on the surface. Such prepared implants were transferred to the Department of Oral Surgery for further tests.

Model of the jaw

Before the decontamination process, each implant was placed in peri-implantitis jaw model. The model was made from acrylonitrile butadiene styrene (ABS) which is a common thermoplastic polymer. According to the cumulative interceptive supportive therapy (CIST) protocol,[12] mechanical debridement and surgical operation classification is needed when the bone loss depth is greater than 5 mm. Following this standard, 6-millimiter bone loss depth was defined in our model. The artificial bone defect was created by removing of the material with the calibrated trephine drill around the implant side.

Decontamination protocols

Every group of implants was decontaminated with 3 different methods. Before the decontamination process, each implant was placed in peri-implantitis model. Different protocols of implant surface decontamination were used in the study:

– Sonic scaler mechanical debridement with a Woodpecker PT5 sonic scaler (Woodpecker, Guilin, China) (s). Each implant was treated with a sonic device for 2 min alone (Fig. 1).

– Mechanical debridement with sonic scaler and with the combination with chemical agent Perisolv® (Regedent AG, Zurich, Switzerland). Each implant was pre-treated with Perisolv® application for 30 s, then sonic scaler was applied for 2 min (s+p) (Fig. 2).

– Er:YAG laser treatment. Implants were decontaminated with Er-YAG (LiteTouch™, Yokneam, Israel) laser irradiation with a 1.3 × 17 mm tip, working up and down continuously for 2 min, and the laser beam parameters were set for 40 mJ, 0.80 W, 20 Hz (Er:YAG) (Fig. 3).

Each implant was treated with the specific method and sent for further microbiological evaluation. The procedure for each implant was repeated 3 times and the results were averaged.

Quantitative evaluation of microorganisms present in the biofilm on the implants surface

Biofilm from the surface of the implants was removed with the use of an aqueous saponin solution. The implants (each separately) were placed in 1 mL of 0.5% saponin solution and shaken for 1 min (2,500 rpm; Heidolph Reax Control ; Heidolph Instruments GmbH & CO. KG, Schwabach, Germany). The obtained suspension of strains (saponin solution and bacteria suspended in it, detached from the surface of the implant) was immediately cultured on McConkey's medium. In the inoculation of bacteria, undiluted suspension was used, and suspension with dilutions from 1:10 to 1:1,000 inoculating volume: 10 L, 20 L, 50 L, and 100 L. In order to obtain maximum separation of the biofilm, the procedure of its removal was repeated 3 times. Inoculated plates with McConkey's medium were incubated at 37°C for 22–24 h.

Fig. 1. Sonic scaler mechanical debridement

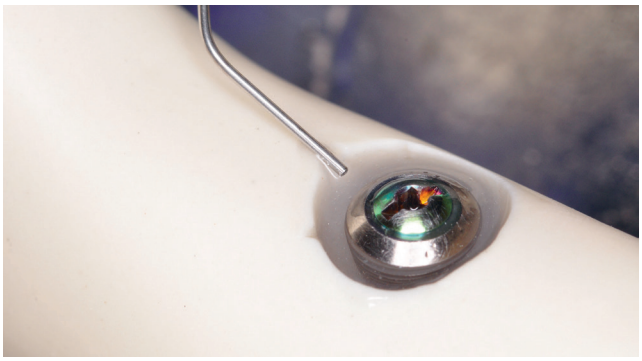

Fig. 2. Application of the Perisolv®

Fig. 3. Er:YAG laser irradiation

Reading the results

After incubation, the colonies grown on the plates were counted and the results obtained were given as the number of colony-forming units (CFU) per 1 mL. The percentage of biofilm reduction R [%] after the tested factor of biofilm removal acted on was calculated according to the formula:

$$R = [(S_C - S)/S_{C]} \bullet 100\%,$$

where S_C (CFU/mL] – the total number of *E. coli* cells detached from the implant coating biofilm without the test factor acting (number of CFU/mL on the control implant);

S (CFU/mL) – the total number of *E. coli* cells detached from the implant coating biofilm, which remain after the test factor acted.

In addition, to compare and reduce the measurement error, the degree of biofilm reduction was calculated after the rejection of extreme values:

$$R' = [(S'_C - S')/S'_{C]} \bullet 100\%,$$

where S'_C (CFU/mL] – the total number of *E. coli* cells detached from the implant coating biofilm without the test factor acting (number of CFU/mL on the control implant), with no maximum or minimum value;

S' (CFU/mL) – the total number of *E. coli* cells detached from the implant coating biofilm, which remain after the test factor acted, with no maximum or minimum value.

Statistical analysis

Two-way analysis of variance (ANOVA) and Tukey's post hoc test were performed. All data is given as means ± standard deviation (SD). A p-value <0.05 was considered statistically significant. The results were analyzed with STATISTICA v. 13 (StatSoft Poland, Kraków, Poland).

Results

The highest level of biofilm reduction (R') for both mechanical and combined mechanical and chemical methods of decontamination was achieved for machined-surface implants (98.66% ±1.19% (for s) and 98.61% ±1.39% (for s+p)) and for SLA implants (96.86% ±2.81% (for s) and 95.23% ±4.68% (for s+p)) (Fig. 4–6). Taking under consideration all of the methods, there were no statistically important differences between decontamination of M and SLA group of implants, although there were statistically important differences between M and HA as well as SLA and HA groups. Surprisingly, as it can be seen, additional application of the chemical agent did not improve decontamination of machined-surface and SLA implants, although it significantly improved the decontamination of HA-coated implants (78.82% ±13.69% (for s) while 85.26% ±19.65% (for s+p)). Also, what is worth reporting are the unstable results in that group of implants (Fig. 6).

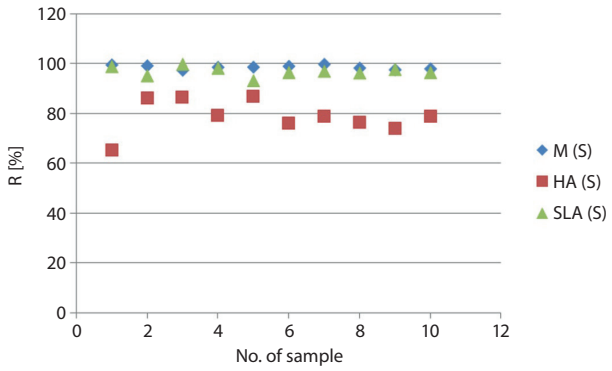

Fig. 4. The results of scaler application on the decontamination of the implant

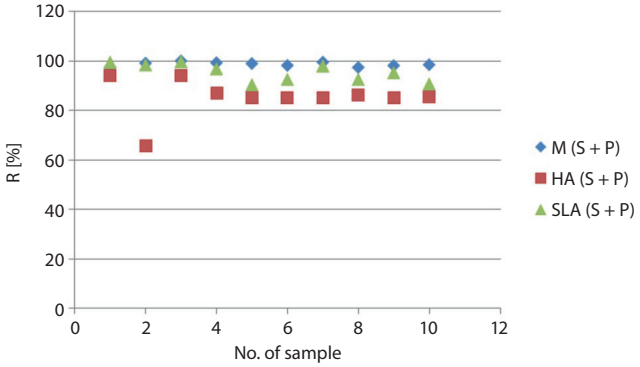

Fig. 5. The results of combined application of scaler and perisolv application on the decontamination of the implant

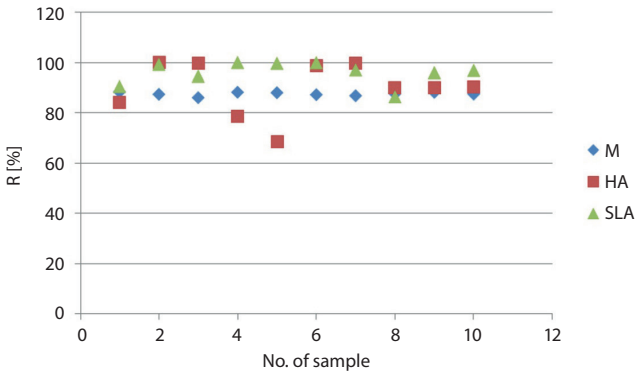

Fig. 6. The results of Er:YAG application on the decontamination of the implant

However, HA-coated implants demonstrate a level of biofilm reduction after Er:YAG irradiation that is better than machined-surface implants and lower than SLA implants (89.99% ±21.52% for HA, 87.40% ±1.49% for M and 95.98% ±5.45% for SLA). Taking under consideration the method of decontamination, the best results were achieved for combined mechanical and chemical (s+p) and this is the treatment of choice for SLA and M surfaced implants. The mechanical debridement reveals comparable to laser irradiation results. Laser application is also the method of treatment for HA implants decontamination.

The intra- and extragroup statistical analysis is shown in Table 1. The statistically important differences are in bold.

Table 1. Differences in the percentage of biofilm reduction between applied methods in relation to the implant surface

Biofilm reduction		M; Scal	M; Scal.+Perisolv	M; Laser Er:YAG	HA; Scaler	HA; Scal.+Perisolv	HA; Laser Er:YAG	SLA; Scal.	SLA; Scal.+Perisolv	SLA; Laser Er:YAG
98.7%	M; Scal.		1.000	**<0.05**	**<0.05**	**<0.05**	**<0.05**	0.997	0.876	0.968
98.6%	M; Scal.+Perisolv	1.000		**<0.05**	**<0.05**	**<0.05**	**<0.05**	0.998	0.885	0.971
87.4%	M; Laser Er:YAG	**<0.05**	**<0.05**		**<0.05**	0.992	0.974	**<0.05**	**<0.05**	**<0.05**
78.8%	HA; Scal.	**<0.05**	**<0.05**	**<0.05**		0.159	**<0.05**	**<0.05**	**<0.05**	**<0.05**
85.3%	HA; Scal.+Perisolv	**<0.05**	**<0.05**	0.992	0.159		0.553	**<0.05**	**<0.05**	**<0.05**
90.0%	HA; Laser Er:YAG	**<0.05**	**<0.05**	0.974	**<0.05**	0.553		0.106	0.413	0.238
96.9%	SLA; Scal.	0.997	0.998	**<0.05**	**<0.05**	**<0.05**	0.106		0.998	0.999
95.2%	SLA; Scal.+Perisolv	0.876	0.885	**<0.05**	**<0.05**	**<0.05**	0.413	0.998		0.999
96.0%	SLA; Laser Er:YAG	0.968	0.971	**<0.05**	**<0.05**	**<0.05**	0.238	0.999	0.999	

M – machined-surface implants; HA – hydroxyapatite-coated implants; SLA – sandblasted and acid-etched implants. Differences statistically important are in bold at $p \leq 0.05$.

Discussion

The methods used to decontaminate the surface of dental implants can be divided into 3 main groups. The 1st group is comprised of the mechanical methods, the 2nd group is comprised of the methods based on application of chemical agent on the implant surface and within the periodontal sulcus, and the 3rd group includes physical methods (e.g., PDT or laser application). The mechanical debridement is also often an introduction to further therapy and is even considered as priority method.[13–15]

Mechanical debridement was also the first method used to manage peri-implantitis. The methods imported directly from the treatment of periodontitis were rather disappointing and resulted in damage to the fragile implant surface. That was the reason why alternative decontamination methods in dental implantology were sought. Mengel et al. were one of the first to evaluate several methods of mechanical debridement and to prove their safe applicability to fragile implant surface in an in vitro study.[7] Blasi et al. provided the evaluation of different mechanical methods including ultrasonic scalers with plastic tips, titanium curettes, and airflow with glycine powder and with rubber cup and polishing paste. The study was conducted on patients suffering from severe (including CIST criteria) peri-implantitis and/or mucosistis. They proved no statistically important difference between all 4 mechanical methods of implant surface decontamination.[16]

Denisson et al. were one of the first to design a study to evaluate the mechanical and chemical decontamination method of the dental implants with different surfaces.[17] The dental implants were coated with radioactive endotoxin (125I-LPS). In an in vitro study, an air-powder abrasive with sodium bicarbonate as well as citric acid solution (CA), or 0.12% CHX was used as a decontamination method on 2 different dental implant surfaces (titanium plasma-sprayed (TPS), HA-coated and machined-surface). As a result, they found the superiority of chemical methods. It was also found that machined-surface implants were decontaminated more effectively than the other surfaces by all treatments. The only exception for this statement was citric acid treatment, which was equally effective on either machined or hydroxyapatite surfaces.

The superiority of the chemical method in comparison to other methods of dental surface decontamination was reconfirmed in the study of Marotti et al. In an in vitro study, SLA implants were contaminated with the saliva collected from patients experiencing peri-implantitis. Several decontamination methods were applied, including the application of 0.12% CHX, GaAlAs laser irradiation (660 nm, 30 mW) for 3 min or 5 min (7.2 J and 12 J) without and with methylene blue dyes in PDT. They achieved greater decontamination in CHX group compared to the laser group and similar to the PDT group.[18]

In other study conducted on SLA implants, the use of Er:YAG laser irradiation resulted in statistically significantly superior biofilm removal compared to the 3 other treatments (titanium curettes, PDT and curettes with adjunctive PDT). The study also proved no statistically important differences in the reattachment of epithelial cells (EC), gingival fibroblasts and osteoblast-like cells to titanium SLA surfaces after each method of decontamination.[19] To the best of our knowledge, there are very few

studies in the field of decontamination which are similar to our work on different surface implants. One of the mentioned studies was carried on SLA, TPS and HA implants. After Er:YAG laser irradiation at pulse energies of 60 mJ and 120 mJ and at a frequency of 10 pps led to bacterial reductions of 99.51% (SA), 98.39% (HA) and 99.6% (TPS) at a pulse energy of 60 mJ, and 99.92% (SA), 99.85% (HA) and 99.94% (TPS) at 120 mJ.[20]

Conclusions

The superiority of combined chemical-mechanical method of decontaminating the surface of an implant on SLA and machined-surface implants was proved. On the contrary, Er:YAG laser irradiation was reported as the best option for decontaminating the HA-coated implants.

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Cerebrovascular reactivity and disease activity in relapsing-remitting multiple sclerosis

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Abstract

Background. In multiple sclerosis (MS), insufficient blood supply might worsen energy deficiency of the brain tissue. Thus, cerebrovascular reactivity (CVR), which is the capacity of cerebral circulation to match blood supply to metabolic demand, might be important in MS pathology.

Objectives. The objective of this study was to investigate the relationship of CVR to disease activity and neuroimaging markers of disease progression in patients with MS.

Material and methods. In 43 patients with relapsing remitting MS (RRMS) in clinical remission, 30 patients with a relapse of MS and 30 healthy controls, we measured CVR with transcranial Doppler as a relative change in flow velocity after breath-holding (breath-holding index) and voluntary hyperventilation (hyperventilation index). All patients in remission underwent brain magnetic resonance imaging at baseline and 33 underwent repeated imaging after 12 months, with various brain volume measurements taken.

Results. Cerebrovascular reactivity indices did not differ between patients in remission, patients with a relapse and controls. In patients in remission, CVR did not differ between those with or without contrast-enhancing lesions. In patients with a relapse, glucocorticoids significantly reduced both CVR indices. Cerebrovascular reactivity was not related to brain volume, white matter lesion volume, percent brain volume change, and the change in total white matter lesion volume.

Conclusions. In RRMS, CVR appeared normal and unrelated to disease activity. There was no substantial association of CVR to brain atrophy and accumulation of white matter lesions.

Key words: multiple sclerosis, brain atrophy, cerebral blood flow, cerebrovascular reactivity, transcranial Doppler ultrasonography

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Introduction

Multiple sclerosis (MS) is an inflammatory disease of the central nervous system, but neurodegenerative mechanisms are also implicated in the pathogenesis of MS.[1] For example, in MS, histotoxic hypoxia may cause energy deficiency of the brain tissues, which promotes demyelination and axonal loss.[2] Energy deficiency in MS might be worsened by impaired regulation of cerebral blood flow. Notably, many studies have found diffusely reduced cerebral blood flow in patients with MS compared with healthy age-matched controls.[3] Moreover, evidence form magnetic resonance imaging (MRI) and histologic studies shows that most white matter (WM) lesions in patients with MS are found in areas with the lowest blood perfusion, the so-called watershed zones.[4–6] Similarly, in an animal model of MS, demyelinating lesions tend to develop in areas with the worst blood supply.[7] Thus, cerebrovascular reactivity (CVR), which is the capacity of cerebral circulation to match blood supply to metabolic demand, might be important in MS pathology. Cerebrovascular reactivity changes might be related to autonomic dysfunction, which is common in MS.[8,9] Usually, CVR is estimated as a relative increase in cerebral flow after increasing the systemic carbon dioxide concentration (CO_2 inhalation, breath-hold).[10,11]

Reduced CVR may cause insufficient energy supply, particularly to areas with an increased energy demand, such as damaged neurons or WM lesions infiltrated by metabolically active immune cells.[12] Cerebrovascular reactivity impairment could amplify axonal loss and demyelination. Indeed, during healthy aging, WM areas with the lowest CVR are most susceptible to demyelination.[13] Similarly, CVR impairment is associated with an increased risk of cerebral ischemia.[14]

To date, few studies have investigated CVR in MS,[15–17] and one group showed that CVR might be reduced in MS.[18,19] It remains unclear, however, whether clinical and neuroimaging disease activity is related to CVR in MS. We also do not know whether low CVR increases the risks of brain atrophy and accumulation of demyelinating lesions. Because previous findings on CVR in MS are inconsistent and come from small studies, we checked whether CVR was impaired in MS in a larger group of patients. Moreover, we related CVR to clinical and neuroimaging disease activity and longitudinal changes in brain volume and WM lesion volume.

Material and methods

Participants

The study included 43 patients with RRMS in clinical remission, for at least 3 months, who received interferon beta in our clinic, 30 patients with RRMS who received intravenous methylprednisolone (1 g for 3–5 days) due to a relapse (an increase in Extended Disability Status Scale (EDSS) score of at least 1 point), and 30 healthy controls matched for age, sex and cardiovascular risk factors (Table 1). The study was approved by the Bioethics Committee of our Institute, and all participants singed informed consent before enrolment.

Assessment of cerebrovascular reactivity

Cerebrovascular reactivity was assessed with transcranial Doppler ultrasonography (TCD) at around noon (11 AM to 2 PM). We recorded blood flow velocity in the middle cerebral artery with a 2-megaherz probe fixed with a headband (DWL, Singen, Germany). Blood flow was monitored in either the left or right middle cerebral artery, whichever had a better signal quality. The mean flow velocity (MFV) was calculated over 10–15 heart cycles. During TCD measurements, we continuously recorded the end-tidal carbon dioxide concentration ($EtCO_2$) with a capnometer (NMed, Beijing, China). The baseline MFV was recorded after about 5 min of resting. Then, we recorded the MFV after 2 min of hyperventilation ($EtCO_2$ had to decrease by at least 10% compared to baseline). After at least 3 min, when the MFV and $EtCO_2$ returned to baseline, we recorded the MFV after 30 s of breath-hold. Cerebrovascular reactivity was estimated with the breath-hold index (BHI) and a CO_2-normalized hyperventilation index ($HV_{\Delta CO2}$) as follows:

$$BHI = \frac{\text{MFV after breat – hold – baseline MFV}}{\text{baseline MFV} \times 30};$$

$$HV_{\Delta CO_2} = \frac{\text{baseline MFV – MFV after hyperventilation} \times 100\%}{\text{baseline MFV} \times \text{EtCO 2 change from baseline to hyperventilation}}.$$

Higher values of both BHI and $HV_{\Delta CO2}$ indicated greater CVR. In patients with a relapse, TCD measurements were taken on the first and last day of intravenous glucocorticoid treatment, before the first and after the last injection, respectively. In patients in remission, TCD measurements were taken within 1 h before MRI. Cerebrovascular reactivity was assessed only once in patients in remission and controls. Atherosclerosis of the carotid arteries was ruled out by color Doppler ultrasound.

Magnetic resonance imaging and image analysis

After the TCD study, patients in remission underwent brain MRI, as part of a routine clinical follow-up. With a 1.5 T scanner (Philips, Eindhoven, the Netherlands), we acquired 3D T1-weighted images, before and after intravenous gadolinium (Gd) injection, (TR, 25 ms; TE, 4.6 ms; field of view, 240 × 240 mm; voxel resolution, 0.937 × 0.937 × 1 mm) and 2D FLAIR images (TR, 11,000 ms; TE, 140 ms; field of view, 0.898 × 0.898 × 3 mm). The presence of Gd-enhancing

(Gd(+)) lesions was assessed by an independent radiologist. Normalized brain volume (NBV) for head size, and normalized volumes of grey matter (GM) and WM were measured based on the T1-weighted images with the SIENAX software.[20] The lesion-TOADS software was used to measure the total volume of WM lesions (TLV) based on the FLAIR and T1-weighted images[21]; TLV was normalized for head size based on scaling coefficients derived from SIENAX. Before running lesion-TOADS, we extracted brains from whole-head images with the SPECTRE tool and registered the T1-weighted images to the FLAIR images (rigid body registration).[21] During the study, 33 out of 43 patients in remission had repeated MRI after 12 months (1 patient entered secondary progressive MS, 3 patients were lost to follow-up, 6 patients had less than 12 months of follow-up). For the 33 patients in remission who had follow-up brain MRI after 12 months, we calculated the percentage brain volume change (PBVC), with the SIENA software, and the TLV change, with lesion-TOADS.[20]

Statistical analysis

Baseline anthropometric and clinical characteristics were compared between the groups of participants with one-way analysis of variance (ANOVA), the Mann–Whitney U test, and Fisher's exact test, as appropriate. In patients with a relapse of MS, the differences in CVR indices before and after intravenous methylprednisolone treatment were compared with the dependent samples t-test. The Mann–Whitney U test was used to compare CVR indices between patients with or without Gd(+) lesions. The Pearson coefficient or the Spearman coefficient (rho) was calculated to study correlations between pairs of variables. A value of $p < 0.05$ was considered significant. All analyses were completed in the statistical package R (www.r-project.org).

Results

Clinical and imaging characteristics

There were no significant differences in age, proportion of women, and the frequency of cardiovascular risk factors between patients with MS in remission, patients with a relapse of MS and controls (Table 1). Among patients with MS in remission, the studied brain volumes correlated negatively with EDSS, and disease duration correlated positively with TLV (Table 2).

Cerebrovascular reactivity

Neither of the 2 CVR indices, i.e., BHI and $HV_{\Delta CO2}$, differed significantly between patients with MS in remission, patients with a relapse of MS before intravenous glucocorticoid treatment and controls ($p = 0.56$ for BHI; $p = 0.1$ for

Table 1. Clinical characteristics of patients with relapsing-remitting multiple sclerosis in remission, patients during a relapse, and controls

Variable	Controls (n = 30)	Remission (n = 43)	Relapse (n = 30)	p-value
Age [years]	37.2 ±8.5	38.2 ±8.9	36.1 ±8.0	0.60[a]
Women, n (%)	23 (77)	33 (77)	24 (80)	0.96[b]
Disease duration [years]	–	6.3 ±4.9	10.1 ±7.3	0.02[c]
Median EDSS (range)	–	1.5 (0–6.0)	4.0 (2.0–4.0)	<0.001[d]
Initial TLV [mL]	–	7.4 ±5.2	–	–
TLV change [mL]	–	0.42 ±1.8	–	–
PBVC	–	−0.36 ±0.49	–	–
SBP [mm Hg]	130.7 ±11.0	133.9 ±15.0	127.7 ±14.2	0.17[a]
DBP [mm Hg]	84.8 ±7.0	87.3 ±10.3	82.4 ±12.0	0.12[a]
Blood glucose [mg/dL]	100.8 ±17.2	98.0 ±20.1	99.0 ±17.8	0.84[a]
Hypertension, n (%)	4 (13)	5 (12)	2 (7)	0.78[b]
Smoking, n (%)	5 (17)	4 (9)	4 (13)	0.65[b]
Dyslipidemia, n (%)	0	2 (5)	1 (3)	0.78[b]
Diabetes mellitus, n (%)	0	0	1 (3)	0.58[b]
Disease-modifying treatments, n (%)	–	IFNβ-1a, 19 (44) IFNβ-1b, 24 (56)	None, 20 (67) GA, 3 (10) IFNβ-1b, 2(7) DMF, 2 (7) TFN, 1 (3) FNG, 1 (3) LQD, 1 (3)	–

The values are means ± standard deviations (SD) unless otherwise specified; a – one-way analysis of variance (ANOVA); b – Fisher's exact test; c – independent samples t-test; d – Mann–Whitney test; EDSS – Extended Disability Status Scale; TLV – total lesion volume; PBVC – percent brain volume change; SBP – systolic blood pressure; DBP – diastolic blood pressure; IFN – interferon; GA – glatiramer acetate; DMF – dimethyl fumarate; TFN – teriflunomide; FNG – fingolimod.

Table 2. Correlations between cerebrovascular indices and clinical and imaging variables in patients with multiple sclerosis in remission (n = 43). Significant correlations are in bold

Variable	NBV	GMV	WMV	TLV	PBVC	ΔTLV
Age	r = –0.47 p = 0.001	r = –0.59 p < 0.001	r = –0.19 p = 0.22	r = 0.25 p = 0.11	r = 0.21 p = 0.25	r = 0.18 p = 0.31
Disease duration	r = –0.29 p = 0.06	r = –0.39 p = 0.01	r = –0.09 p = 0.58	r = 0.34 p = 0.03	r = 0.01 p = 0.99	r = 0.03 p = 0.86
EDSS	rho = –0.34 p = 0.03	rho = –0.29 p = 0.06	rho = –0.31 p = 0.04	rho = –0.05 p = 0.73	rho = 0.05 p = 0.78	rho = 0.28 p = 0.11
TLV	r = –0.46 p = 0.002	r = –0.53 p < 0.001	r = –0.25 p = 0.11	–	r = –0.16 p = 0.38	r = 0.44 p = 0.01
BHI	r = 0.21 p = 0.17	r = 0.10 p = 0.50	r = 0.10 p = 0.50	r = –0.03 p = 0.85	r = –0.12 p = 0.52	r = –0.1 p = 0.59
$HV_{\Delta CO2}$	r = 0.15 p = 0.33	r = 0.13 p = 0.41	r = –0.03 p = 0.86	r = –0.08 p = 0.61	r = –0.21 p = 0.25	r = 0.12 p = 0.50

NBV – normalized brain volume; GMV – grey matter volume; WMV – white matter volume; TLV – total lesion volume; PBVC – percent brain volume change; ΔTLV – change in total lesion volume; EDSS – Extended Disability Status Scale; BHI – breath-hold indices; $HV_{\Delta CO2}$, CO_2 – normalized hyperventilation indices. All brain volumes were measured in milliliters.

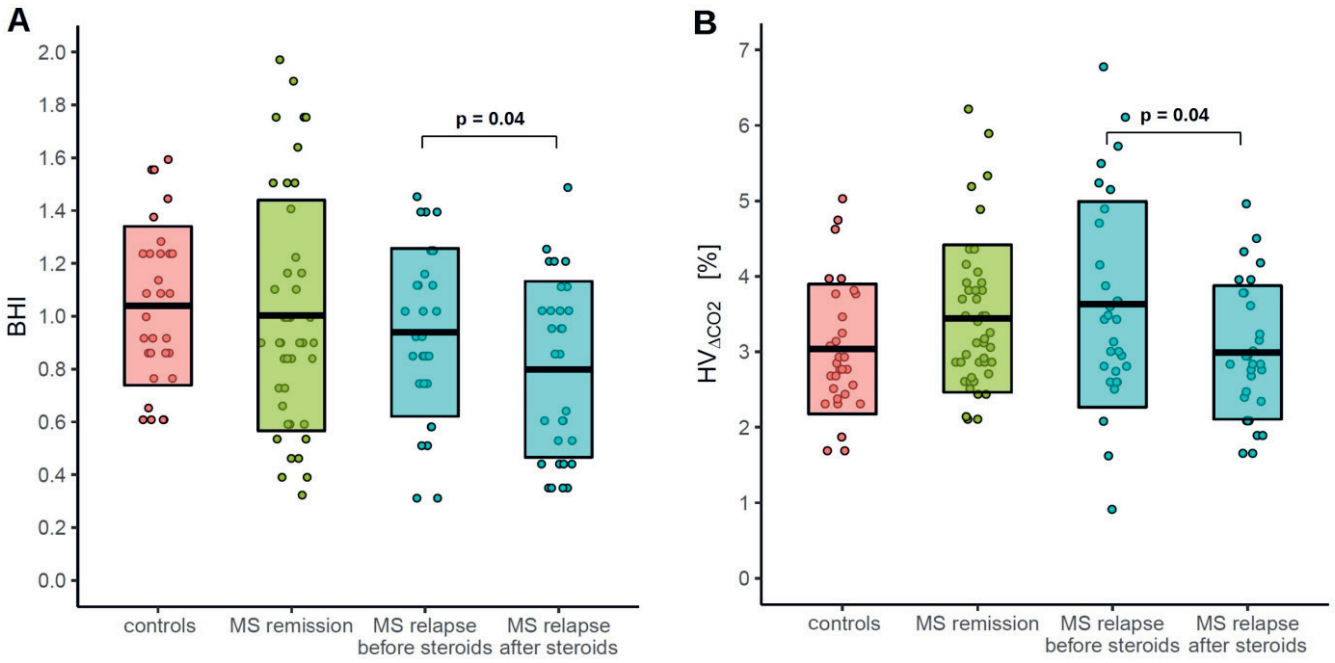

Fig. 1. (A) Breath-hold indices (BHI) and (B) CO_2-normalized hyperventilation indices ($HV_{\Delta CO2}$) in healthy controls (n = 30), patients with multiple sclerosis (MS) in remission (n = 43), and patients with a relapse of MS before and after intravenous glucocorticoid treatment (n = 30). The middle bar represents the mean, and the upper and lower bars represent standard deviations (SD)

$HV_{\Delta CO2}$; Fig. 1). In patients with a relapse of MS, however, both CVR indices decreased significantly after intravenous glucocorticoid treatment (p = 0.04 for BHI and $HV_{\Delta CO2}$; Fig. 1).

Among patients with remission, BHI and $HV_{\Delta CO2}$ did not differ between those with (n = 10) or without (n = 33) Gd(+) lesions (p = 0.20 for BHI, and p = 0.81 for $HV_{\Delta CO2}$). Among patients in remission, neither of the 2 CVR indices correlated with the brain volumes studied, TLV, PBVC, and TLV change (Table 2). Similarly, BHI and $HV_{\Delta CO2}$ did not correlate significantly with EDSS or disease duration in patients in remission or relapse (data not shown).

Discussion

Our findings suggest that CVR is normal in RRMS and that it does not change during a relapse of MS or in patients with Gd(+) lesions. However, we found that treatment with intravenous glucocorticoids reduced CVR in patients with a relapse of MS. In patients with MS in clinical remission, CVR was not related to any of the brain volume measures, including the longitudinal change in brain volume and WM lesion volume. Thus, it seems that there is no substantial relationship between CVR and diseases activity and neuroimaging markers of disease progression in RRMS.

Our findings are in line with those in most previous studies, which have reported normal CVR in patients with MS. In the study by Uzuner et al. (n = 12), CVR measured with TCD did not differ between patients with RRMS and controls. In contrast to our study, those investigators did not find any significant effect of glucocorticoids on CVR.[16] In another TCD-based study, Khorvash et al. reported that CVR was higher in RRMS than in patients with migraines. However, that study did not include healthy controls.[15] Similar to our findings, Metzgen et al., who measured CVR with blood oxygen level-dependent (BOLD) functional MRI (fMRI) after CO_2 inhalation, observed normal CVR in MS and did not find CVR to correlate with brain volume and WM lesion volume.[17] Those investigators showed that CVR was reduced in patients with MS and cognitive impairment, but this relationship is found in other diseases as well.[22] To date, based on arterial spin labeling (ASL) fMRI, only 1 group has reported reduced CVR in MS. Moreover, that group found that CVR correlated negatively with WM lesion volume and GM atrophy.[18] ASL-based fMRI may be the best method to study CVR impairment in MS; however, CVR measurements based on ASL and BOLD fMRI usually lead to similar conclusions.[23,24] Moreover, in Alzheimer's disease, reduced CVR has been demonstrated with many techniques, including BOLD, ASL and TCD.[25]

Marshall et al. hypothesized that CVR might be reduced in MS due to habituation of cerebral vasculature to chronically increased nitric oxide concentrations.[18] However, nitric oxide seems essential for hypercapnia-induced cerebral vasodilation,[26] and consequently for CVR, and we were not able to find any previous evidence that nitric oxide, when chronically increased, like in MS,[27] has an opposing effect.[28,29] In contrast, scavenging of nitric oxide by reactive oxygen species (ROS) reduces vasodilation, which could occur in MS.[30,31]

Different effects of inflammation, such as increased oxidative stress, might reduce CVR. For example, in patients with diabetes, higher concentrations of inflammatory markers were associated with reduced CVR.[32] In our study, CVR was similar in patients with clinical remission and a relapse of MS, and Gd(+) lesions were not associated with reduced CVR. However, we did not measure inflammatory markers in our study. We observed that treatment with glucocorticoids, which have anti-inflammatory effects, not only did not improve CVR, but significantly reduced it. Similarly, in patients with diabetes, reduced CVR was associated with increased concentrations of endogenous cortisol.[32] We suspect that the glucocorticoid-induced reduction of CVR might be due to a direct effect of glucocorticoids on cerebral vessels. For instance, glucocorticoids decrease endothelial synthesis of nitric oxide, and they increase the sensitivity of vascular smooth muscle cells to endogenous vasoconstrictions, such as norepinephrine.[33] However, we measured CVR twice in patients with a relapse only and not in those in remission or in controls. Therefore, the effect of glucocorticoids on CVR that we observed might be due to physiological variability or becoming familiar with the procedure by participants.

Because CVR is a measure of cerebral metabolic reserve, we suspected that reduced CVR would be related to greater brain atrophy and greater accumulation of WM lesions, particularly because most WM lesions in MS occur in areas with reduced blood flow and reduced CVR.[4–6,13] However, in our study, CVR was not related to brain volume reduction and the change in WM lesion volume.

Our study had limitations. First, it included a relatively small group of patients. However, with over 70 patients with RRMS, our study is the largest study on CVR in MS to date. Additionally, the included number of patients allowed us to observe the well-established relationships in MS, such as the correlation between EDSS, disease duration, and brain volume. Because we did not include patients with progressive MS, our findings may not hold true for these patients. Second, we included patients with MS in remission who received interferon beta only. Although the effect of interferon beta on CVR is unknown, a study among 5 patients with MS showed that interferon beta increased blood flow in the basal ganglia.[34] Moreover, interferon beta, similar to other disease-modifying treatment, slows the rate of brain atrophy and lesion accumulation in MS.[35] Thus, the potential relationship between CVR and brain atrophy along with lesion accumulation might be abolished by treatment with interferon beta. Third, some investigators regard breath-holding a less reliable hypercapnic stimulus than CO_2 inhalation.[36] Others, however, have found these 2 stimuli equivalent for estimating CVR.[37] Moreover, fMRI might be better than TCD for measuring CVR, but there is a good agreement between these 2 approaches.[38] In addition to breath-holding, we used voluntary hyperventilation to measure CO_2-normalized CVR. The relationships between CVR and other variables in our study were consistent when assessed with the 2 CVR indices (BHI, $HV_{\Delta CO2}$). Apart from the use of 2 vasoactive stimuli to measure CVR, the strengths of our study include enrolment of patients in remission and a relapse of MS, measurement of CVR before and after glucocorticoid treatment, and longitudinal MRI analyses. We also measured CVR in all participants at the same time of the day, because CVR may decrease by over a third from morning to evening.[29]

We conclude that CVR is normal and is not related to disease activity in patients with RRMS. Moreover, CVR seems unrelated to the accumulation of WM lesions and brain atrophy in these patients. It would be worthwhile to verify our findings with fMRI-based CVR measurements, preferably in larger studies that would enroll patients with progressive MS.

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The clinical utility of remote ischemic preconditioning in protecting against cardiac surgery-associated acute kidney injury: A pilot randomized clinical trial

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Abstract

Background. Cardiac surgery-associated acute kidney injury (CSA-AKI) is a well-known, serious complication and a well-recognized independent risk factor for higher morbidity and mortality among patients undergoing cardiac surgery.

Objectives. The aim of the study was to assess the efficacy of remote ischemic preconditioning (RIPC) in reducing the incidence of CSA-AKI, measured with the standard creatinine technique and using neutrophil gelatinase-associated lipocalin (NGAL) serum concentrations as a potential new biomarker of kidney damage. The ethics committee of the Medical University of Lodz prospectively approved the protocol (approval No. RNN/286/13/KE). The study was retrospectively registered with the U.S. National Institutes of Health – NIH (29 June 2017; ClinicalTrials.gov identifier: NCT03205410).

Material and methods. We conducted a prospective single-center double-blind randomized and controlled tudy. Data was collected from patients admitted to the Cardiosurgery Clinic at the Medical University of Lodz (Poland) between January and December 2014, scheduled for elective cardiac surgery (an off-pump coronary artery bypass). A total of 28 patients were randomized to receive either RIPC (n = 14) or sham RIPC (n = 14). After the induction of anesthesia, the patients assigned to the RIPC group underwent 3 cycles of 5-minute inflation to 200 mm Hg and 5-minute deflation of the upper-arm cuff. The control group had a deflated cuff placed on the upper arm for 30 min. The authors measured the patients' serum creatinine concentration to check for the occurrence of a CSA-AKI within 48 h after cardiac surgery, and NGAL serum concentration to check its level within 3 h after the operation.

Results. Fewer patients in RIPC group developed CSA-AKI within 48 h after cardiac surgery than in the control group (29% vs 93%; p = 0.003). Fewer patients in the RIPC group presented an increase in NGAL 3 h after surgery (medians: 124 vs 176.7; p = 0.0003).

Conclusions. In patients undergoing an off-pump coronary artery bypass, RIPC significantly reduces the occurrence of CSA-AKI and protects against increased postoperative NGAL levels.

Key words: neutrophil gelatinase-associated lipocalin, remote ischemic preconditioning, cardiac surgery-associated acute kidney injury

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Acute kidney injury (AKI) is a well-known, serious complication and well-recognized independent risk factor of higher morbidity and mortality in patients undergoing cardiac surgery[1,2]; it is even referred to as cardiac surgery-associated acute kidney injury (CSA-AKI).[3] Approximately 30% of patients develop AKI after cardiac surgery[4] and 1–5% of AKI patients require dialysis therapy.[1,5] The CSA-AKI can be caused by a variety of factors and in different combinations, including ischemia and reperfusion injury, toxins, metabolic abnormalities, neurohormonal activation, inflammation, and oxidative stress.[6] Although preventing AKI after cardiac surgery would improve survival, there are still no efficient methods.[7,8] The current definition of AKI is based on serum creatinine concentration (SCr) and urine output, and is described as any of the following: an increase in SCr ≥ 0.3 mg/dL (≥26.5 lmol/L) within 48 h; or an increase in SCr to ≥1.5 times baseline, which is known or presumed to have occurred within the prior 7 days; or urine volume <0.5 mL/kg/h for 6 h.[9] Both SCr and urine volume are markers of renal function but not kidney injury.[10] Furthermore, according to the definition, AKI can be diagnosed using the creatinine technique after at least 2 days. This has led to investigations of new AKI biomarkers that could show kidney injury much earlier, within a few hours. During the past few decades several potential biomarkers of AKI have been identified, including neutrophil gelatinase-associated lipocalin (NGAL),[11] kidney injury molecule 1 (KIM-1),[12] interleukin 18 (IL-18),[13] liver-type fatty acid-binding protein (L-FABP),[14] tissue inhibitor of metalloproteinase 2 (TIMP-2), insulin-like growth factor-binding protein 7 (IGFBP7),[15] calprotectin,[16] and urine microRNAs.[17] Neutrophil gelatinase-associated lipocalin is by far the most investigated and most promising, especially as an early AKI biomarker. Fast identification of AKI is very important, as is appropriate implementation of preventive strategies, which are the most effective tools to improve AKI outcome.[18]

Remote ischemic preconditioning (RIPC) is a phenomenon in which non-lethal periods of alternating ischemia and reperfusion applied to tissue or an organ can remotely protect another. At first, RIPC was known as a cardioprotection method,[19] but it has also turned out to be effective in distant organs such as kidneys, offering protection in kidney transplantation[20] or contrast-induced AKI,[21] and seems promising in preventing AKI in patients who have undergone cardiac surgery.[22,23] However, its efficacy still remains controversial.[24] The mechanism of RIPC is complex and not well understood. Several triggers, intracellular pathways, humoral and neural effectors, as well as effectors induced by genetic changes may be considered potential pathways in the protective activity of RIPC.[25]

We conducted this prospective randomized controlled clinical study to assess whether RIPC reduces the incidence of AKI measured with the standard Scr technique and using neutrophil gelatinase-associated lipocalin (NGAL) as a potential new biomarker of kidney damage. The aim of our investigation was to analyze the safety and clinical outcomes of RIPC after elective isolated primary off-pump coronary artery bypass graft surgery (OPCAB).

Material and methods

Study design

This was a prospective single-center double-blind randomized controlled study. The ethics committee of the Medical University of Lodz (Poland) approved the protocol, and the study was conducted in accordance with the Helsinki Declaration and national law. Written informed consent was provided by all participants before enrollment in the study. The study design, along with the data collection and analysis, was conducted solely by the authors.

The patients

From January 2014 to December 2014, we screened patients over 18 years of age who were admitted to the Cardiosurgery Clinic of the Medical University of Lodz and scheduled for elective cardiac surgery (OPCAB). Enrollment was non-consecutive and dependent on whether one of the investigators who enrolled participants was available. Exclusion criteria were a history of cardiac surgery, acute myocardial infarction up to 7 days before surgery, chronic stage 4 or 5 kidney disease (estimated glomerular filtration rate (eGFR) <30 mL/min/1.73 m^2), peripheral vascular disease affecting the upper limbs, a history of severe injuries and operations within 2 months before cardiac surgery, a history of cancer, chronic autoimmune diseases, and dialysis. Patients were recruited during their preadmission consultations.

Experimental protocol

Following the placement of intravenous and right radial artery catheters and after the induction of anesthesia, the patients were randomly assigned in a 1:1 ratio to either the RIPC group or the control group by means of a computerized randomization table. A blinded investigator who was not involved in either the surgery or the randomization procedure performed RIPC in the RIPC group or sham RIPC in the control group. The RIPC group underwent 3 cycles of 5-minute inflation to 200 mm Hg followed by 5-minute deflation of the left upper-arm cuff (in excess of contralateral systolic radial artery pressure). The control group had a deflated cuff placed on the left upper arm for 30 min. Remote ischemic preconditioning took place after the induction of anesthesia and was completed prior to skin incision.

Surgical and anesthetic procedures

Prescribed cardiac medications were administered up to the evening preceding surgery. Beta-adrenergic receptor

antagonists were given on the day of the surgery, while agents that can interfere with RIPC (e.g., sulphonylurea, angiotensin-converting enzyme inhibitors, angiotensin receptor blockers) were transiently withdrawn 24 h before the operation. All the patients were given standardized nephroprotective procedures such as the withdrawal of potentially nephrotoxic agents 24 h before surgery and hydration by intravenous fluid infusion according to their clinical state, using the following formula: 60 mL of balanced solutions + 1 mL per every kilogram of body weight over 20 kg per hour (i.e., approx. 1.5–2 mL/kg/h of balanced solutions) for 4 h prior to the surgery; and in patients with congestive heart failure or eGFR < 30 mL/min/1.73 m^2: infusion of 1 mL/kg/h of balanced solutions for 12 h prior to the surgery.

Anesthesia was induced with intravenous propofol (1 mg/kg), fentanyl (3.5 μg/kg) and pancuronium (0.1 mg/kg) and maintained with prolonged infusion of propofol (0.01–0.02 mg/kg/min) and fentanyl (0.05 μg/kg/min). All the patients were mechanically ventilated in controlled mechanical ventilation mode with 50% oxygen concentration. The surgical procedure was performed through median sternotomy according to standardized protocols. Postoperative fluid management in all the patients was performed according to clinical state of the individual patient and Enhanced Recovery After Surgery guidelines[26]: 1.5 mL/kg/h on the day of the surgery, reduced the day after to 70 mL/h. Fluid therapy was conducted by monitoring central venous pressure, invasive blood pressure, pulse pressure variation, systolic pressure variation, urine volume, and the daily fluid balance. Serum osmolarity was maintained in the range of 280–305 mOsm/kg H_2O. Fluid delivery included crystalloids (using balanced solutions excluding 0.9% NaCl and 5% glucose[26]). All of the participants were operated on by the same surgical team, and postoperative care was performed by the same anesthesiologist. The average duration of the surgery was 206 min in patients who received RIPC (median: 172.5 min; interquartile range (IQR) = 155–260 min) vs 187 min in the control group (median: 177.5 min; IQR = 130–235 min). The difference in duration between the 2 groups was not statistically significant (p = 0.037).

Blood sampling and analysis

Venous blood samples were drawn before surgery and at 3 h and 48 h after surgery for measurement of serum creatinine and NGAL concentrations. Serum creatinine levels were measured with an enzymatic assay (Crea Creatinine OSR6578; Beckman Coulter Inc., Brea, USA). We used an enzyme-linked immunosorbent assay (ELISA) test to measure NGAL concentrations (Human Lipocalin – 2/NGAL ELISA; BioVendor Laboratory Medicine Inc., Brno-Řečkovice, Czech Republic). Estimated glomerular filtration rate was calculated using the Cockcroft–Gault formula.

Endpoints

The primary endpoint of the study was the incidence of AKI within 48 h after cardiac surgery or increased NGAL level within 3 h after the operation. Acute kidney injury was classified according to the Kidney Disease Improving Global Outcomes (KDIGO) criteria as any of the following: (1) an increase in SCr ≥ 0.3 mg/dL (≥26.5 lmol/L) within 48 h after surgery; or (2) an increase in SCr to ≥1.5 times baseline, which is known or presumed to have occurred within the previous 7 days; or (3) urine volume <0.5 mL/kg/h for 6 h after surgery.

Secondary endpoints were the length of hospitalization, the length of intensive care unit (ICU) stay, ventilation time, the occurrence of postoperative atrial fibrillation (AF), the time of renal replacement therapy (RRT), and death from any cause.

Statistical analysis

We performed the statistical analysis using the STATISTICA v. 10 software (StatSoft Polska Sp. z o.o., Kraków, Poland). For all the tests, we used p = 0.05 as the threshold of statistical significance. The Shapiro–Wilk normality test was used to verify the distribution assumptions for normality. Categorical variables are represented as the number of observations (N) and the corresponding percentages (%). Quantitative variables are presented as median and IQR. Pearson's χ^2 test was used to check group equality. If the number of cases was less than 5, Yates's correction for continuity was used. The distribution of most of the variables under consideration was not normal. Continuous variables that were not distributed normally were analyzed with a nonparametric test. In order to compare 2 independent trials, the Mann–Whitney U test was used. For a comparison of 2 repeated measurements between 2 matched samples of continuous variables, we used the Wilcoxon signed-rank test. To detect differences in continuous values across multiple test attempts, we used Friedman's test.

Results

Study population characteristics and operative data

A total of 58 patients were assessed for eligibility, with 30 patients excluded before randomization due to an exclusion criteria or consent withdrawal. This left 28 patients who were enrolled and randomized to receive either RIPC (n = 14) or sham RIPC (n = 14) and included in the primary analysis (Fig. 1). The preoperative characteristics and intraoperative protocols were similar in the 2 groups (Table 1). Preoperative serum creatinine and NGAL concentrations were also similar in the 2 groups. The time between the end of the last inflation of the blood-pressure cuff and the skin incision was 6 ±1 min.

Table 1. Characteristics of the study population

Characteristics	RIPC (n = 14)	Control (n = 14)	p-value
Age [years]	65 (60–71)	67 (61–72)	0.804
Sex (%)	M: 9 (64) F: 6 (36)	M: 8 (57) F: 6 (43)	0.699
BMI [kg/m^2]	31 (29.1–32.3)	27.7 (25.4–30.9)	0.062
Systolic blood pressure [mm Hg]	112.5 (105–120)	120 (120–130)	0.077
Diastolic blood pressure [mm Hg]	70 (65–70)	70 (70–80)	0.164
GFR [mL/min/1.73 m^2]	108 (82–118)	73.5 (55–105)	0.062
SCr [µmol/L]	72.5 (61–82)	85.5 (69–108)	0.21
Serum NGAL concentration [ng/mL]	127.1 (102.3–139.5)	102.3 (68.2–139.5)	0.227
CCS class, n (%)	2.5 (2–3)	2 (2–2)	0.227
NYHA class, n (%)	2 (2–3)	2.5 (2–3)	0.839
History of heart attack, n (%)	7 (50)	7 (50)	1
History of stroke/transient ischemic attack, n (%)	2 (14)	0 (0)	0.463
Current smoking, n (%)	9 (64)	7 (50)	0.445
Hypertension arterialis, n (%)	14 (100)	13 (93)	0.309
Chronic heart failure, n (%)	4 (29)	4 (29)	0.676
Dyslipidemia, n (%)	11 (79)	12 (86)	0.622
IGT/IFG, n (%)	0 (0)	1 (7)	1.00
Diabetes mellitus, n (%)	5 (36)	8 (57)	0.256
COPD, n (%)	1 (7)	3 (21)	0.59
Chronic kidney disease, n (%)	1 (7)	2 (14)	1.00
Nephrolithiasis, n (%)	1 (7)	2 (14)	1.00

Data is presented as median (interquartile range) or n (%). M – male; F – female; RIPC – remote ischemic preconditioning; BMI – body mass index; GFR – glomerular filtration rate; SCr – serum creatinine concentration; NGAL – neutrophil gelatinase-associated lipocalin; CCS – Canadian Cardiovascular Society; NYHA – New York Heart Association; IGT – impaired glucose tolerance; IFG – impaired fasting glucose; COPD – chronic obstructive pulmonary disease.

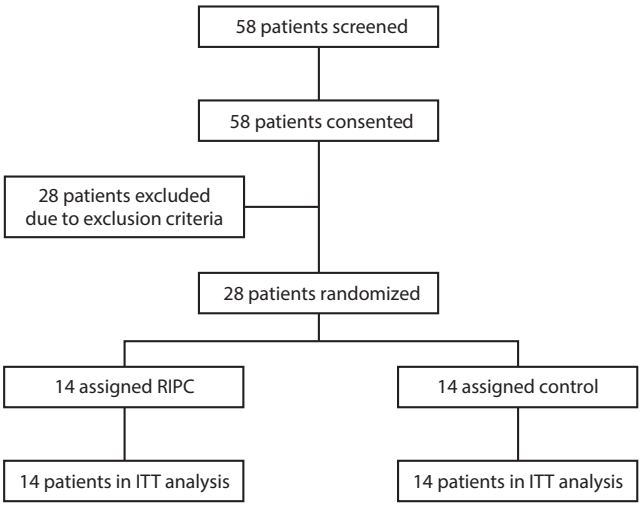

Fig. 1. Flowchart of the patients in the study

RIPC – remote ischemic preconditioning; ITT – intention-to-treat analysis.

Primary outcomes

Significantly fewer patients in the RIPC group developed AKI within 48 h after cardiac surgery compared with the control group (Table 2), with absolute risk reduction of 0.64. Similarly, the patients in the RIPC group presented significantly lower serum NGAL concentrations 3 h after surgery compared to the control group (Table 2). Moreover, the patients who received RIPC showed either a decrease or only a slight increase in serum NGAL levels compared to the control group, who manifested significant increases in NGAL levels (Fig. 2).

Secondary outcomes

Serum creatinine concentration (SCr), tested on admission, did not differ between the 2 groups (p = 0.21; Table 1). However, analysis of SCr over time – on admission, 48 h after OPCAB and on discharge – showed that in the patients who received RIPC before cardiac surgery, SCr did not change statistically (p = 0.147; Fig. 3). In contrast, the patients in the control group showed significantly different levels of SCr over time (p = 0.0004; Fig. 3). Likewise, GFR was not significantly different in the 2 groups at baseline (p = 0.062; Table 1). Glomerular filtration rate changes over time were not significantly different in the RIPC group as opposed to the control group (p = 0.374 vs p = 0.0499; Fig. 4). However, we found no significant differences between the groups in terms of the time of receiving mechanical ventilation (p = 0.756), the length of their stay

Table 2. Operative and postoperative history of the study participants

Variable	RIPC	Control	p-value
Occurrence of AKI (%)	4 (29)	13 (93)	**0.003**
GFR on admission [mL/min/1.73 m^2]	108 (82–118)	73.5 (55–105)	0.062
GFR 48 h after surgery [mL/min/1.73 m^2]	95.5 (67–137)	47.5 (36–69)	**0.005**
GFR on discharge [mL/min/1.73 m^2]	98.5 (81–141)	72 (70–107)	0.062
SCr on admission [µmol/L]	72.5 (61–82)	85.5 (69–108)	0.21
SCr 48 h after surgery [µmol/L]	79.5 (70–125)	130.5 (102–158)	**0.014**
SCr on discharge [µmol/L]	70 (60–84)	75 (71–93)	0.454
NGAL on admission [ng/mL]	127.1 (102.3–139.5)	102.3 (68.2–139.5)	0.227
NGAL 3 h after surgery [ng/mL]	124 (111.6–142.6)	176.7 (155.0–204.6)	**0.0003**
Hospitalization [days]	11 (10–14)	10 (9–13)	0.454
ICU stay [days]	2.5 (2–8)	3 (2–3)	0.667
Ventilation time [days]	1 (1–1)	1 (1–1)	0.756
Time of RRT [days]	0 (0–0)	0 (0–0)	0.982
Postoperative AF (%)	2 (14)	5 (36)	0.383
Death (%)	1 (7)	0 (0)	1.00

Data is presented as median (interquartile range) or n (%). RIPC – remote ischemic preconditioning; AKI – acute kidney injury; GFR – glomerular filtration rate; SCr – serum creatinine concentration; NGAL – neutrophil gelatinase-associated lipocalin; ICU – intensive care unit; RRT – renal replacement therapy; AF – atrial fibrillation.

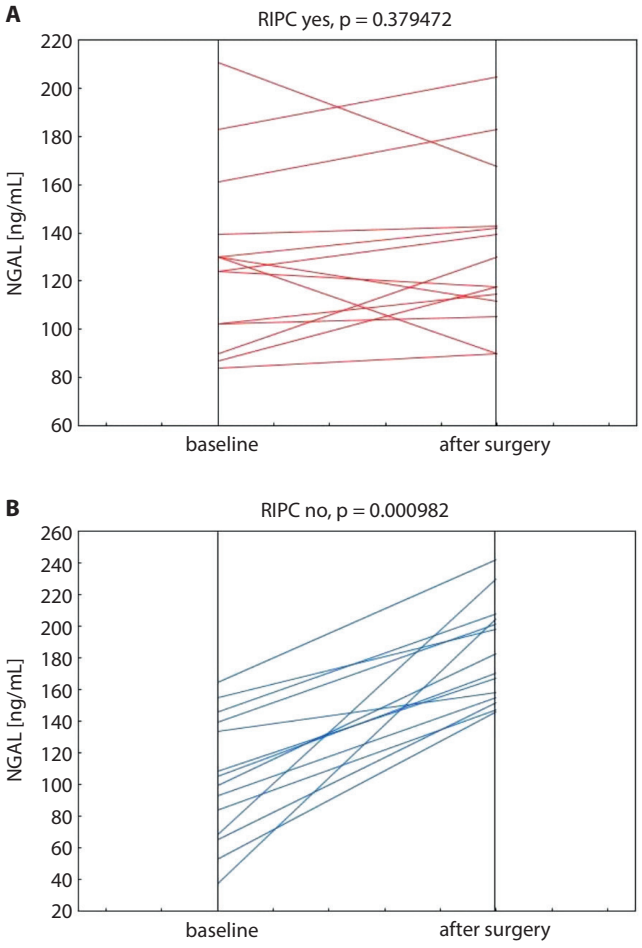

Fig. 2. NGAL serum concentration increase (difference between baseline and 3 h after surgery) in (A) the patients who received RIPC and (B) the control group (no RIPC)

NGAL – neutrophil gelatinase-associated lipocalin; RIPC – remote ischemic preconditioning.

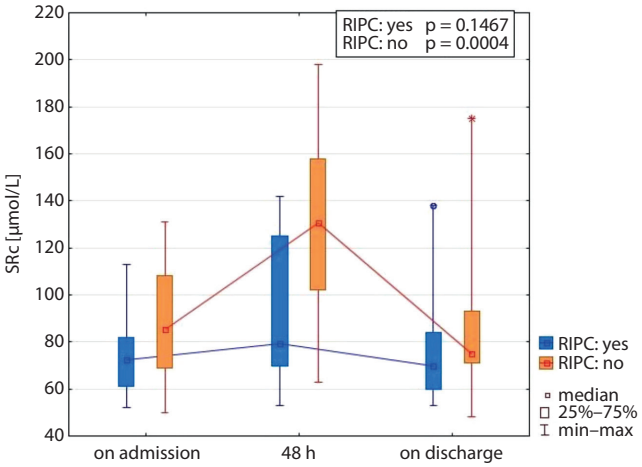

Fig. 3. Variability of serum creatinine concentration [µmol/L] over time (admission to the Cardiosurgery Clinic, 48 h after surgery, discharge from the hospital) in the RIPC group vs the control group (no RIPC)

SCr – serum creatinine concentration; RIPC – remote ischemic preconditioning.

in the ICU (p = 0.667), the length of their hospital stay (p = 0.454), the occurrence of postoperative AF (p = 0.383), or death (p = 1.00). In each group, 1 patient required dialysis in the postoperative period, and there were no significant differences in the length of renal replacement therapy (p = 0.982).

Discussion

Cardiac surgery patients have a high risk of AKI. Simultaneously, the development of AKI is associated with higher mortality and a higher risk of complications in patients

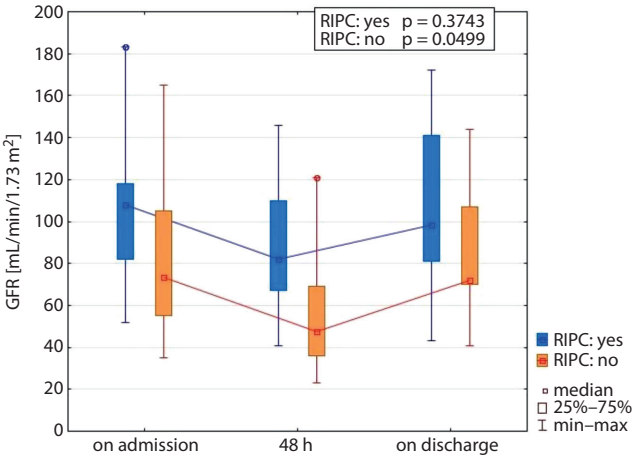

Fig. 4. Variability of GFR [mL/min/1.73 m^2] over time (admission to the Cardiosurgery Clinic, 48 h after surgery, discharge from the hospital) in (1) the RIPC group vs (0) the control (no RIPC) group

eGFR – estimated glomerular filtration rate; RIPC – remote ischemic preconditioning.

undergoing cardiac surgery.[27] However, there are no effective clinical strategies for preventing AKI. Remote ischemic preconditioning holds promise as a simple and inexpensive way of protecting tissues against ischemic damage, including kidney protection, which has led to research into the use of this method to prevent AKI. Nowadays, the standard diagnostic tools for AKI detection, such as SCr and urine output monitoring, are markers of renal function but not kidney injury. Furthermore, SCr depends on various intrarenal and extrarenal functions and its concentration characterizes the balance between creatinine generation and excretion.[28] Serum creatinine concentration is a delayed and insensitive biomarker of changes in kidney function, and its concentration does not differentiate the triggers of kidney failure and could be affected by many factors.[29] Damage biomarker such as NGAL may quickly allow cellular kidney damage to be identified and lead to earlier diagnosis of AKI. Although NGAL is represented in some human tissues, it is one of the most upregulated transcripts in the kidney after ischemic, toxic or septic AKI in animal and human models, implying that it has a role as an early marker of structural renal tubular damage.[30]

In our single-center double-blind study involving 28 patients at a high risk of postoperative AKI, RIPC did reduce the prevalence of AKI, according to KDIGO criteria, based on increases in SCr. Our surprising finding that 93% of the control group as well as 29% of the RIPC group developed AKI may result from the small number of participants as well as the sensitivity of the KDIGO AKI definition, which is based on only a slight elevation in SCr. Furthermore, we showed the benefit of RIPC, with reduced levels of SCr and higher GFR 48 h after OPCAB. In the patients who received RIPC prior to surgery, only a 9.66% increase in SCr level compared to the baseline was observed. In contrast, in the patients without RIPC, the postoperative SCr level increased significantly, by as much as 52.63%. Remote ischemic preconditioning turned out to be protective against significant increases in SCr as well as decreases in GFR over time. Moreover, we found that the postoperative expression of NGAL, an early biomarker of AKI, was significantly reduced in patients who underwent RIPC.

Even though the prevalence of AKI was lower in the RIPC group, our study found no benefits of RIPC in terms of the length of ICU stay, the duration of mechanical ventilation or length of hospitalization. This may be due to the small study group. Although fewer patients in the RIPC group showed postoperative AF, the overall assessment showed no significant differences.

The effect of RIPC on kidney function differs among studies. Our findings are consistent with the randomized controlled trial by Zarbock et al.[31] Their study was specifically designed and powered to look at the effect of RIPC on AKI as the primary endpoint. As in our research, they noticed a significant absolute risk reduction in the incidence of AKI in the RIPC group, and higher postoperative NGAL levels in the control group ($p = 0.04$). Furthermore, in a meta-analysis including 26 trials, the rate of AKI was significantly lower in the RIPC groups than in the control groups among patients undergoing cardiac and vascular interventions ($p = 0.001$; RR = 0.79).[32] However, it should be noticed that various definitions of AKI were used in different studies. The same report found no benefits of RIPC in postoperative SCr and eGFR levels, in-hospital mortality, initiation of RRT, or the length of hospital stay. This is consistent with a similar meta-analysis where postoperative incidence of AKI was significantly reduced by RIPC ($p = 0.02$), but no benefit was found in terms of renal replacement therapy and mortality.[33] A recent meta-analysis including 27 randomized trials also showed that RIPC lowers the risk not only of acute renal failure, but also myocardial infarction, stroke and composite risk of all-cause mortality; however, statistically the results were only marginally significant.[34] The recently published results of the 90-day follow-up of the RenalRIP trial showed that RIPC improves short- as well as long-term outcomes of high-risk patients undergoing cardiac surgery.[35] In that study, RIPC clearly reduced the occurrence of major adverse kidney events at 90 days (including all-cause mortality, RRT and persistent renal dysfunction without dialysis), compared with the controls. Also, considering different components of composite endpoints, persistent renal dysfunction and RRT were significantly higher in the patients that did not undergo RIPC.[35]

On the other hand, some trials reported that RIPC did not lead to any significant difference in clinical outcomes compared to the controls. In an 11-center randomized controlled trial involving patients at high risk of AKI and undergoing cardiac surgery, RIPC yielded no demonstrable benefits. The median peak of postoperative change in creatinine was not statistically significant (absolute

mean difference: 0.06, 95% confidence interval (95% CI) = 0.10–0.23).[36] Likewise, in the RIPValve study, in patients with aortic valve stenosis who underwent elective aortic valve replacement, RIPC also had no impact on postoperative renal function.[37] Two large multicenter double-blind randomized controlled trials where propofol was used to maintain anesthesia noted no benefits of RIPC. The Remote Ischemic Preconditioning for Heart Surgery (RIPHeart) study and the Effect of Remote Ischemic Preconditioning on Clinical Outcomes in Patients Undergoing Cardiac Surgery (ERICCA) study investigated clinical outcomes in patients undergoing cardiac surgery.[38,39] Neither of them showed any evidence of positive effects of RIPC on death within 12 months, postoperative AF, AKI, postoperative release of NGAL, or the duration of ICU and hospital stay.[40] The use of propofol anesthesia in more than 90% of the patients of ERICCA and all the patients in RIPHeart is the most plausible explanation for the failure of RIPC to provide protection.[41]

Similarly, the presence of diabetes mellitus may impair conditioning-mediated protection.[42] Despite the fact that 36% of the RIPC group and 57% of the control group in our study presented diabetes mellitus, we found that RIPC protected against the development of CSA-AKI. Possible explanations for the differences in findings may include differences in the patient populations, the duration of RIPC and, of course, the small sample size in our study.

Limitations

Our study has some limitations. It is a single-center trial with a relatively small sample size, and although we have found important associations with intermediary endpoints, we cannot prove the mechanism. Also, enrollment in the study depended upon the availability of the investigator, which could have biased the sample. It may also have contributed to our surprising finding that 93% of the control group and 29% of the RIPC group developed AKI, which distinguishes our study from the literature. Possible explanations for these differences may be the small sample size of our study, but also the fact that GFR at admission is almost significantly lower ($p = 0.062$) in the control patients compare to the patients enrolled in the RIPC group.

Conclusions

In patients undergoing OPCAB, RIPC significantly reduces the occurrence of CSI-AKI and limits SCr increase over time. The extremely easy-to-apply, low-cost and non-invasive nature of RIPC makes it an ideal method for the prevention of AKI. The introduction of RIPC strategy into widespread clinical settings for the benefit of patients undergoing heart surgery could represent a promising and simple strategy to provide additional protection of kidney function and improve postoperative outcomes. Remote ischemic preconditioning may become "the future of nephroprotection" in cardiac surgery. The same applies to the RIPC-mediated postoperative NGAL reduction noted in our pilot trial. Neutrophil gelatinase-associated lipocalin is one of the best biomarkers of AKI, due to its quick release after tubular damage. It opens a new era of earlier detection and prognosis prediction for AKI, compared to the standard definition. It also creates an urgent need to come to an agreement about the cutoff value of NGAL, which should help in redefining AKI according to NGAL levels. Apart from its limitations, our study demonstrated the important role RIPC plays in protecting against AKI after cardiac surgery. Hence, further studies are needed to redefine the clinical utility of RIPC in current practice and to obtain more evidence of its potential benefits.

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Non-Hodgkin lymphoma after liver and kidney transplantation in children. Experience from one center

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Abstract

Background. Post-transplantation lymphoproliferative disorder (PTLD) is a complication of organ transplantation and a life-threatening condition. Children who underwent organ transplantation are at risk of developing lymphoproliferative disorders and, among them, non-Hodgkin lymphoma (NHL) is the most serious.

Objectives. The objective of this study was to describe the clinical course of NHL after liver and kidney transplantation.

Material and methods. Retrospective analysis of medical records of children who underwent liver/kidney transplantation and developed NHL.

Results. Nine children were identified, all girls, 6 after liver and 3 after kidney transplantations. Age at transplantation ranged from 1 year to 13 years (median: 4 years), while age at lymphoma diagnosis from 4 to 17 years (median: 12 years). Time from transplantation to lymphoma diagnosis ranged from 7 months to 12 years (median: 9 years). All but 1 patient developed mature B-cell lymphoma, 4 children – diffuse large B-cell lymphoma (DLBCL), 2 children – Burkitt's lymphoma, 1 child – mature B-cell leukemia, 1 child – Burkitt-like lymphoma, while 1 patient was diagnosed with T-cell lymphoblastic lymphoma. High levels of Epstein–Barr virus (EBV) DNA were found in blood of 3 patients, and EBV in tissue samples was detected in 4 patients. Six patients presented with stage III and 2 with stage IV disease. Two patients had graft involvement. Three children received chemotherapy according to R-CHOP, 3 LMB protocol (2 with addition of rituximab), while 1 received CHOP and 5 courses of COP. T-cell lymphoma patient was treated with Euro-LB protocol. Six out of 8 treated patients are alive with a median follow-up of 6 years. Two children died from disease progression during treatment and 1 from cerebral herniation before starting therapy. All patients experienced at least 1 toxic episode of grade 3 and 4 according to Common Toxicity Criteria Adverse Event (CTCAE). Complications of chemotherapy were manageable and there were no transplanted organ failures.

Conclusions. Our study provides further data on the treatment and outcome of monomorphic PTLD and indicates that it is feasible to treat solid organ recipients with multiagent chemotherapy.

Key words: children, PTLD, non-Hodgkin lymphomas, post-transplantation lymphoproliferative disorders

Introduction

Post-transplantation lymphoproliferative disorders (PTLD) are one of the most severe complications of organ transplantation in children and adults. Post-transplantation lymphoproliferative disorders is a life-threatening condition and the cause of mortality and morbidity in this group of patients. It may cause graft loss. In the 2016 World Health Organization (WHO) classification revision of lymphoid neoplasms, 6 types of PTLD are distinguished: from the most benign, non-destructive lymphoplasmacytic to polymorphic destructive proliferations which do not yet fulfill lymphoma criteria, and the last 2 types include monomorphic PTLD with histopathological features of non-Hodgkin lymphoma (NHL) and rarely occurring classical Hodgkin lymphoma.[1] Children who underwent solid organ transplantation are at significant risk of developing lymphoproliferative disorders and, among them, NHL is the most serious.[2] Treatment of such patients is challenging with regard to the choice of chemotherapy protocol and management of chemotherapy-related complications in an already vulnerable population. We report herein a retrospective analysis of 9 patients with post-transplant NHL who were treated in our center, the only one in Poland where kidney and liver transplantations in children are performed.

Material and methods

Among 1,518 patients who underwent liver (618 patients) or kidney (900 patients) transplantations in our institution between 1984 and 2015, 9 patients were diagnosed with monomorphic PTLD NHL. Medical records of these patients were reviewed and analyzed for the following data: sex, age at transplantation and NHL diagnosis, type of organ transplanted, indication for transplantation, Epstein–Barr virus (EBV) status at the time of transplantation and NHL diagnosis, type of immunosuppression, previous history of PTLD, time from transplantation to NHL, NHL subtype, disease stage, graft organ involvement by lymphoma, treatment, its toxicity, and outcome.

The diagnosis of lymphoma was based on a histological and immunohistochemical examination of tumor tissue and was classified according to WHO classification. Non-Hodgkin lymphoma was staged according to Revised International Pediatric Non-Hodgkin Lymphoma Staging System.[3] Epstein–Barr virus DNA has been quantified in the routine follow-up of immunosuppressed transplanted patients to detect EBV reactivation or primary infection. It was performed with polymerase chain reaction (PCR) in serum or whole blood and in the biopsy sample of a tumor using EBV-encoded RNA in situ hybridization. Treatment toxicity was graded according to Common Toxicity Criteria for Adverse Events v. 4 (CTCAE) and reported after each chemotherapy cycle.[4]

Due to small number of heterogenous patients, no statistical analyses including regression analysis were performed.

Results

All patients were girls, aged from 1 to 13 years (median 4 years) at the time of transplantation and 4 to 17 years (median: 12 years) at lymphoma diagnosis. Six of them (1%) underwent liver and 3 (0.33%) kidney transplantation. Indications to liver transplantation were biliary atresia in 5 patients and acute liver failure from mushroom poisoning in 1. Kidney transplantations were performed for end-stage kidney disease.

Initial post-transplantation immunosuppression consisted of tacrolimus and prednisone (3 patients); tacrolimus, cyclosporine, mycophenolate mofetil, and prednisone (1 patient); cyclosporine and rapamycin (1 patient); cyclosporin, rapamycin, azathioprine and prednisone (1 patient); cyclosporin, rapamycin, mycophenolate mofetil, and prednisone (1 patient); or rapamycin, mycophenolate mofetil and prednisone (1 patient). One patient received induction with anti CD25 antibody (daclizumab) in addition to tacrolimus, mycophenolate mofetil and prednisone. Since none of the patients experienced severe acute organ rejection episodes, there was no use for anti-thymocyte globulin.

Time from transplantation to lymphoma diagnosis ranged from 7 months to 12 years (median: 9 years). One child 5 years after liver transplantation and 4 years prior to lymphoma diagnosis experienced polymorphic PTLD episode, which was successfully managed with the reduction of immunosuppression and 2 courses of rituximab (375 mg/m^2).

All but 1 patient developed mature B-cell lymphoma, 4 children (50%) – DLBCL, 2 – Burkitt's lymphoma, 1 – mature B-cell leukemia, 1 – Burkitt-like lymphoma, and 1 patient was diagnosed with T-cell lymphoblastic lymphoma. Very high numbers of EBV DNA copies (53,000/mL, 105,000/mL, 150,000/mL, respectively) were found in peripheral blood and serum of 3 patients at the onset of lymphoma – PTLD, whereas EBV was detected in tissue samples in 4 patients with mature B-cell lymphoma. Eight patients had advanced disease, 6 of them presented with stage III and 2 with stage IV (1 lymphoma/leukemia, and 1 patient with massive central nervous system (CNS) disease (Fig. 1). Two patients presented with lymphoma graft involvement – of kidney and liver.

Clinical characteristics of patients with NHL following liver/kidney transplantation are presented in Table 1.

Reduction of immunosuppression (RIS) or discontinuation was undertaken in all 9 patients at PTLD diagnosis without satisfactory response. One girl received 2 courses of rituximab, after which disease progression was observed. Two out of 4 children with DLBCL received 6 courses of R-CHOP. One was treated with group C lymphoma malignancy B-cell (LMB) protocol. Three patients

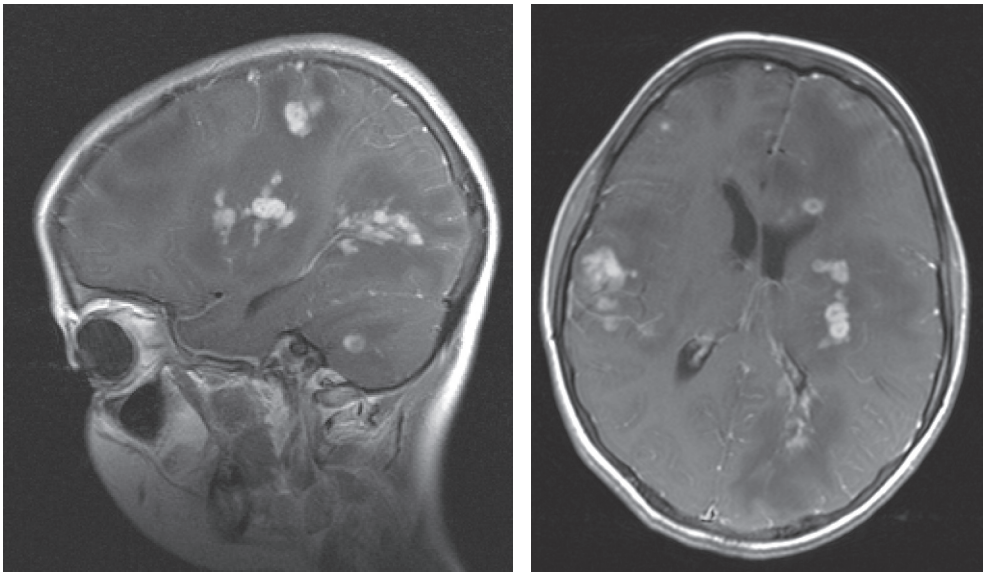

Fig. 1. CT scans showing numerous enhancing lesions throughout both cerebral hemispheres – patient No. 2

Table 1. Patients' characteristics

Patient No.	Age [years] at transplantation/ year of transplantation	Indication for transplantation/ organ/donor	Age [years] at NHL diagnosis/year of NHL diagnosis	NHL type stage/ primary site	EBV status: serum/tumor (copies/mcg DNA in serum)	Treatment protocol	Outcome survival time from NHL diagnosis
1	2 1998	biliary atresia/ liver/living donor	11 2007	Burkitt's lymphoma/III lymph nodes, abdomen/ mediastinum, orbital mass	+/– (10,823)	LMB 89+R, AHSCT	ADF 11 years
2	8 1998	biliary atresia/ liver/living donor	17 2007	DLBCL/IV CNS, lungs, spleen	+/+ (1,532)	prednisone	died from cerebral herniation 3 days
3	4 2001	biliary atresia/ liver/living donor	4 2001	DLBCL plasmoblastic type/III lymph nodes – abdomen	–/+	LMB chemo-therapy dose reduction	died from PD 11 months
4	3 2002	biliary atresia/ liver/deceased	15 2014	DLBCL/III bowel	+/+ (400)	R-CHOP	ADF 4 years
5	1 2002	biliary atresia/ liver/living donor	11 2012	Burkitt-like lymphoma/III oral cavity, tonsils submandibular and mediastinal lymph nodes	–/–	R-CHOP	ADF 6 years
6	13 2007	mushroom poisoning/liver/ deceased	16 2010	TLBL/III infiltration of lungs alone	–/–	EURO LB	ADF 8 years
7	7 2005	end-stage kidney disease/kidney/ deceased	16 2014	DLBCL/III lymph nodes mediastinum/ retroperitoneum	–/–	R-CHOP	ADF 4 years and 2 months
8	4 2011	end-stage kidney disease/kidney/ deceased	8 2015	Mature B-cell leukemia bone marrow, lymph nodes/abdomen	+/+ (53,950,000)	LMB 2001+R	died from PD
9	5 2010	end-stage kidney disease/kidney/ deceased	11 2016	Burkitt's lymphoma/II neck, supraclavicular, mediastinal lymph nodes	+/+ (105,000)	1 course of CHOP 5 courses of COP	ADF 2 years

DLBCL – diffuse large B-cell lymphoma; TLBL – T-cell lymphoblastic lymphoma; LMB – lymphoma malignancy B-cell; EURO-LB – European Intergroup EURO-LB02 protocol; CHOP – cytoxan, vincristine, doxorubicin, prednisone; ADF – alive disease-free; PD – progressive disease; AHSCT – autologous hematopoetic stem cell transplantation; CNS – central nervous system.

with Burkitt's lymphoma received treatment according to LMB and 1 patient received 1 course of CHOP followed by 5 courses of COP chemotherapy. A T-cell lymphoma patient was treated according to European Intergroup EURO-LB02 protocol. All but 2 patients received chemotherapy without modifications of anticancer drugs

dosages. Six out of 9 patients are alive and disease-free after 2–11 years (median follow-up of 6 years). Among them, 1 girl with Burkitt's lymphoma who did not achieve CR during LMB protocol underwent high-dose chemotherapy followed by autologous hematopoetic stem cell rescue. To date, no other PTLD episodes have been observed in this group.

Two children died from disease progression during treatment. One was a girl with stage III DLBCL, who was our first patient with monomorphic PTLD after liver transplantation. Her treatment started with RIS followed by 2 courses of rituximab. Disease progression was observed and LMB protocol applied with reduction of drugs doses. The other deceased patient was a girl with mature B-cell leukemia after kidney transplantation. At PTLD diagnosis, she presented with very high EBV viral load in serum and tumor tissue. Transient response to chemotherapy according to LMB protocol for group C was achieved, then massive progression of leukemia was observed. The 3rd deceased patient was a liver recipient with rapidly progressing CNS lymphoma who died from cerebral herniation before treatment was commenced. All patients experienced at least 1 episode of grade 3 and 4 CTCAE hematologic toxicities episodes during treatment. Grade 3 and 4 hematological toxicity with severe neutropenia and thrombocytopenia was noted in patients following COPADM and CYVE courses. Patients who received rituximab did not experience severe infectious complications and did not require immunoglobulin substitution.

All patients experienced at least 1 episode of neutropenic fever, 6 of them more than once. Oral mucositis was the second most common toxicity. There were 6 grade 3 and 4 episodes after COPADM chemotherapy attributed to methotrexate and doxorubicin. One patient after R-CHOP protocol developed deep vein thrombosis. There were no episodes of transplanted organ failure and no rejections. No unexpected adverse events were observed. All complications were manageable. There were no treatment-related sepsis nor deaths.

Discussion

Children who underwent solid organ transplantation have a higher risk of cancer as compared to the general population. In a study by Yanik et al., among 17,958 pediatric recipients cancer was diagnosed in 392 patients, of which 71% developed NHL. The incidence rate of NHL in this study was 1.3% for both liver and kidney recipients.[5] Others report that the incidence of PTLD varies between 5% and 15% for liver and between 1.9% and 10% for kidney-transplanted children.[6–10] In our patient group, the incidence rate of NHL for liver and kidney recipients was 0.97% and 0.33%, respectively, which is in line with data presented by Yanik et al.

In general, the incidence of PTLD in children is lower for kidney than for liver transplantations,[11] which was also documented in our study. It is most likely that the higher incidence of PTLD in children after liver transplantation, as compared to kidney transplantation, is due to the younger age of liver recipients and their seronegativity to EBV at the time of transplantation, which left them susceptible to primary EBV infection.

Post-transplantation lymphoproliferative disorder in immunocompromised, T-cell-impaired transplant recipients is strongly associated with EBV, which is reflected by EBV viral load in peripheral blood. In our series, 5 out of 9 patients presented with elevated levels of EBV; however, it was of significant value in only 3 patients. The post-solid-organ transplantation EBV-related PTLD appears to be bimodal, with first peak occurring during the first year from transplantation, then years later.[12]

Only 1 of our patients developed PTLD – DLBCL within 1 year after liver transplantation and the child was negative for EBV. For the remaining 7 patients, PTLD – DLBCL development occurred during the 1st decade and for 1 patient 12 years after transplantation. In the study by Yanik et al, which included 278 NHL patients, the median time from transplantation to NHL diagnosis was 1 year and 6 months, which was different than in the studies of other authors and in our data. Her data seems most reliable considering the large number of presented patients.

It is postulated that patients with late onset of PTLD have a more aggressive disease and worse outcome,[13] whereas in our series only 1 patient had an early onset of lymphoma and presented an unfavorable course of disease.

Among transplant recipients diagnosed with NHL, more than 75% develop mature B-cell lymphoma, of which 65% are DLBCL and 9% BL, opposite than in general pediatric population. A few PTLDs are of T-cell origin.[5] This was also demonstrated in our patients; all but 1 had a diagnosis of mature B-cell lymphoma, though the distribution of DLBCL and BL was similar. Most interestingly, 1 of our patients developed primary pulmonary T-cell lymphoma 3 years after liver transplantation. Primary pulmonary lymphoma is a very rare condition and accounts for less than 1% of all NHL cases, of which 80% are of B-cell origin. To the best of our knowledge, very few such cases have been published, none in the pediatric population.[14]

Post-transplantation lymphoproliferative disorder can affect many organs, presenting with non-specific clinical features and extranodal involvement.[15] It has been shown in some studies that it has a propensity to occur in the anatomic region of the transplanted organ.[16,17] In our series, 6 patients had lymph node involvement below and above diaphragm, whereas the remaining 3 had extranodal disease involving the lungs, CNS and intestine. In the general population, about 70% of children with NHL present with advanced stage III and IV disease, which is also true for monomorphic PTLD.[18,19] All of our children had advanced disease, of whom 1 had extranodal massive CNS involvement and another lymphoma/leukemia.

Optimal treatment of PTLD has not yet been established, though the National Comprehensive Cancer

Network (NCCN) or other clinical practice guidelines have been published.[20–22] First-line management of EBV-PTLD involves reducing immunosuppression, and even its discontinuation, which may result in PTLD remission. This particularly concerns patients with less aggressive disease morphology. Rituximab, although unauthorized, is in practice a standard first-line treatment for EBV-PTLD with responses reported in the 44–66% range.[23,24] There is still a substantial number of patients who will fail to respond to rituximab, relapse or present with EBV-negative PTLD, for whom the usual treatment is multiagent chemotherapy with standard treatment protocols specific for lymphoma type. Treatment of adult patients is usually based on chemoimmunotherapy consisting of rituximab and CHOP regimen.[25–27] Gross et al. in a phase II trial demonstrated that rituximab with low-dose cyclophosphamide and prednisone is safe and effective in pediatric patients with EBV, CD20 (+) PTLD. After 2 years, the overall survival rate was 85%.[28] Same efficacy of rituximab with low-dose chemotherapy was confirmed by Gupta et al. and it is a preferable method of treatment.[29]

Our approach was more aggressive in terms of chemotherapy.

The justification for choosing regular chemotherapy regimens was based on the patient's EBV and clinical status, disease severity as well as the time when PTLD was diagnosed. Moreover, treatment failure of our first PTLD patient who did not respond to RIS, had progressive disease after 2 rituximab courses and received unsuccessful chemotherapy with reduced doses of anticancer drugs influenced our decision to treat the following monomorphic PTLD according to current lymphoma protocol, though each case is usually considered individually.

Long-term outcome of monomorphic PTLD in children is estimated to be over 80%.[11,30] Patients with CNS and bone marrow involvement have inferior outcomes as compared to patients with lower disease stage as NHL in general population.[31] This was also observed in our study. In our series, 6 out of 8 treated patients are alive with a long-term follow-up from 2 to 11 years (median: 4 years). To date, none of them neither relapsed nor experienced another PTLD episode. We included in our analyses an untreated patient to demonstrate that PTLD involving the central nervous system can have a fulminant, fatal course not giving enough time for implementing proper treatment.

It is suggested that patients with EBV-negative tumors fare worse than EBV-positive ones.[10] In our study, 4 children with EBV-negative tumors are alive.

Children undergoing treatment of NHL experience chemotherapy-related complications. The most common are hematologic toxicities, neutropenic fever and mucositis. Of special concern are patients with PTLD in whom chemotherapy complications may cause graft failure.[30] During the whole treatment process, none of our patients had any kind of graft insufficiency. Toxicity episodes were as expected following NHL chemotherapy and were all manageable. There were no episodes requiring treatment in the intensive care unit. Moreover, treatment with rituximab did not add adversely to infectious complications.

Our study provides further data on the treatment and outcome of monomorphic PTLD and indicates that it is feasible to treat children with multiagent chemotherapy after solid organ transplantation. The authors acknowledge the limitations of the study, its retrospective character and small number of patients from one institution. However, the presented study comes from a single pediatric-only national transplant center with a large number of performed liver and kidney transplantations, established protocols and multidisciplinary approach, thus making the data reliable for the pediatric population.

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Serum concentration of osteopontin and interleukin 17 in psoriatic patients

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Abstract

Background. Psoriasis is a chronic, autoinflammatory disease characterized by activation and differentiation of naive T lymphocytes towards T helper CD4+ (including Th1 and Th17) and T cytotoxic CD8+. Osteopontin (OPN), which plays an important role in both physiological processes and inflammatory, neoplastic and autoimmune diseases, is also considered in the context of psoriasis pathogenesis. Current data indicates that OPN is a multifunctional protein involved in the modulation of Th1 and Th17 cellular responses, in stimulating keratinocyte proliferation, and in the regulation of cellular apoptosis.

Objectives. The assessment of OPN and interleukin 17 (IL-17) concentrations in the peripheral blood of psoriatic patients in comparison to healthy volunteers as well as the correlations of OPN and IL-17 with the severity of psoriasis.

Material and methods. The study included 107 male psoriatic patients and 41 age-matched healthy men. The serum concentrations of IL-17 and OPN were examined using the enzyme-linked immunosorbent assay (ELISA) method. The skin change severity of psoriasis was assessed using the Psoriasis Area and Severity Index (PASI), Body Surface Area (BSA), Physician Global Assessment (PGA), and Dermatology Life Quality Index (DLQI).

Results. Psoriatic patients had significantly higher concentrations of OPN (31.65 ng/mL on average) than the healthy volunteers (11.42 ng/mL on average) ($p < 0.001$). Interleukin 17 was also higher in psoriatic patients (0.53 pg/mL on average) compared to healthy volunteers (0.09 pg/mL on average) ($p < 0.001$). There was no significant correlation between OPN and IL-17 concentrations in psoriatic patients and in healthy volunteers. Psoriasis severity correlated positively to IL-17 serum concentration, but not to OPN.

Conclusions. Although the study did not show a relationship between OPN and IL-17 concentrations in psoriatic patients, it should be emphasized that serum concentrations were significantly higher in the patients with psoriasis compared to healthy volunteers.

Key words: psoriasis, osteopontin, interleukin 17

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Introduction

Psoriasis is an autoinflammatory disease characterized by a chronic skin inflammation with infiltrations containing T lymphocytes, neutrophils and macrophages.[1–3] Activated Th17 cells, formed from naive CD4+ cells under the influence of interleukin 23 (IL-23),[4] migrate to the skin, where in the presence of pro-inflammatory cytokines, such as IL-1β, they produce IL-17[4] which is known to play a key role in the development of psoriatic plaque.[5]

Another protein, osteopontin (OPN), supposedly engaged in the pathogenesis of psoriasis, has a substantial role in certain physiological processes as well as in the pathogenesis of inflammatory disease, cancer and autoimmune diseases. Osteopontin, first described in 1979 by Senger et al.,[6] is one of the glycoproteins of the SIBLING non-collagen protein family.[7,8] Initial studies on OPN focused on its role in osteogenesis and cancer metastases,[9] while recent studies have been mainly concerned with the effect of OPN on cell migration and autoinflammatory mechanisms.[9] Some data indicates that in chronic systemic inflammation, the increase in peripheral blood OPN concentration may contribute to the development of atherosclerosis and metabolic syndrome as well as psoriasis.[10] Presently, OPN is indicated as a multifunctional protein involved in the modulation of Th1 and Th17 responses and keratinocytes' proliferation as well as in cell apoptosis regulation.[7,11] Osteopontin is also supposed to play a role in the development of autoimmune diseases since it is capable of stimulating macrophages to produce IL-12 and interacting with the CD44 receptor as well as inhibiting IL-10 production, which enables differentiation of T cells into Th1.[7,12–14] As a result of these processes, the concentration of IL-27 decreases, which in turn favors differentiation of T cells into Th17.[7] Due to interaction with αvβ3 integrin, OPN stimulates Th17 to produce IL-17.[7,12,14] In addition, OPN increases the production of interferon gamma (IFNγ) by T cells and IL-6 by monocytes, thereby promoting adhesion and migration of lymphocytes.[12,13] Similar immunological phenomena are also observed in the pathogenesis of psoriatic lesions, which is suggestive of a possible contribution of OPN to the development of local and systemic inflammation in psoriasis.

Nevertheless, the studies on OPN conducted so far, both in the peripheral blood and psoriatic plaque, have produced conflicting results; therefore, the role of OPN in the pathogenesis of psoriasis still awaits elucidation.

Objectives

In light of the recent study results indicative of a stimulating role of OPN in the Th17 response, the aim of our study was to assess the concentration of OPN and IL-17 in the serum of psoriatic patients as well as the correlation between OPN and IL-17 concentrations in the serum and the severity of psoriasis measured with the Psoriasis Area and Severity Index (PASI), Body Surface Area (BSA), Physician Global Assessment (PGA), and Dermatology Life Quality Index (DLQI).

Material and methods

The study was conducted in 2018 and included 107 male psoriatic patients hospitalized in the Department of Dermatology, Venereology and Pediatric Dermatology as well as 41 age-matched healthy men. All the study subjects signed informed consent and gave information about the duration of psoriasis, family history, comorbidities, and addictions. The patients underwent physical examination and were evaluated for psoriasis severity using PASI, BSA, PGA, and DLQI.

In order to determine serum OPN and IL-17 concentrations, peripheral blood samples were collected from both psoriatic patients and healthy volunteers. Blood samples were centrifuged for 15 min at 1,000 × g. Then, serum samples were stored at –80°C until tested. The serum concentrations of OPN and IL-17 were examined with the use of enzyme-linked immunosorbent assay (ELISA) kits (R&D SYSTEMS® Quantikine®ELISA Human Osteopontin, R&D SYSTEMS® Quantikine®HS ELISA Human IL-17) (R&D Systems, Inc., Minneapolis, USA).

The data was statistically analyzed using STATISTICA v. 13.0 (StatSoft, Inc., Tulsa, USA) software. Minimum and maximum values, median and interquartile range (IR) were estimated for continuous variables, as well as the absolute numbers (n) and percentages of the occurrence of items for categorical variables.

We used statistical tests as follows:

– Mann–Whitney U test to compare age as well as OPN and IL-17 serum concentrations between psoriatic patients and healthy volunteers;

– Mann–Whitney U test to compare OPN and IL-17 serum concentrations between psoriatic patients with PASI below 25 and between 25 and 45;

– Pearson's correlation coefficient r to correlate: OPN and IL-17 serum concentrations, OPN serum concentration and psoriasis severity, IL-17 serum concentration, and psoriasis severity;

– Kruskal–Wallis H test to compare OPN and IL-17 serum concentrations between 3 groups of psoriasis severity measured with PGA.

The significance level was assumed at 0.05.

Results

Characteristics of psoriatic patients and healthy volunteers

The study included 107 male patients aged 18–77 years, 47 years on average. The age of the health volunteers was 48 years on average and did not significantly differ from

the psoriatic patients (p = 0.899). The age of psoriasis onset ranged from less than 1 year to 65 years, 25 years on average. Psoriasis duration was from a few months to 48 years, 19 years on average.

In the patients studied, the severity of psoriatic skin lesions expressed with PASI was between 3 and 45, 13 on average. The PASI was ≤5 in 14% of the patients, up to 10 in 17.76%, i.e., 31.78% of the patients had PASI ≤10, whereas 68.22% of them had PASI >10. In the group studied, 12 patients (11.21%) had PASI ≥25.

The extent of the psoriatic skin lesions expressed by BSA in the patients was from 5% to 90%, 25% on average; 25.23% of the patients had BSA ≤10, whereas 74.77% were above 10.

Dermatology Life Quality Index was from 1 to 29 points, 12 points on average. Physician Global Assessment was minimal in 13 patients (12.15%), mild in 57 patients (55.74%), moderate in another 35 individuals (32.71%), and severe in 2 patients (1.87%).

Comparison of osteopontin and interleukin-17 serum concentrations between psoriatic patients and healthy volunteers

Osteopontin serum concentration in the psoriatic patients was in the range of 0.15–185.88 ng/mL, 31.65 ng/mL on average, with IR = 16.79. This was significantly higher than in the healthy volunteers (0.09–21.22 ng/mL, 11.42 ng/mL on average, with IR = 5.90) (p < 0.001, Fig. 1).

Interleukin 17 serum concentration in the psoriatic patients was in the range of 0.10–18.31 pg/mL, 0.53 ng/mL on average, with IR = 0.73. This was significantly higher than in the healthy volunteers (0.05–0.92 pg/mL, 0.09 ng/mL on average, with IR = 0.10) (p < 0.001, Fig. 2).

A correlation between OPN and IL-17 serum concentrations was not found, either in the psoriatic patients (r = 0.144, p = 0.139) or in the healthy volunteers (r = –0.107, p = 0.501).

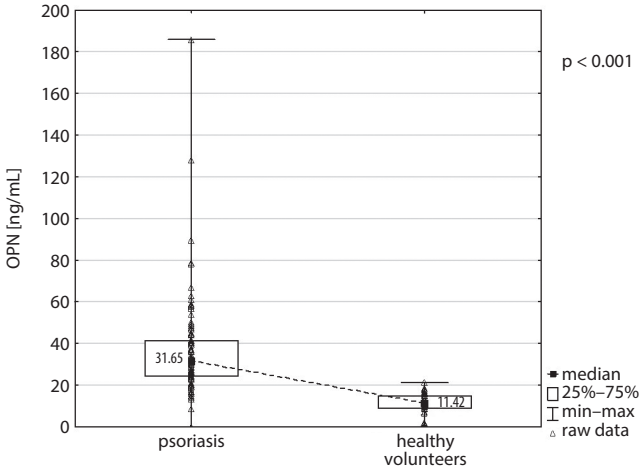

Fig. 1. Comparison of OPN serum concentration between psoriatic patients and healthy volunteers

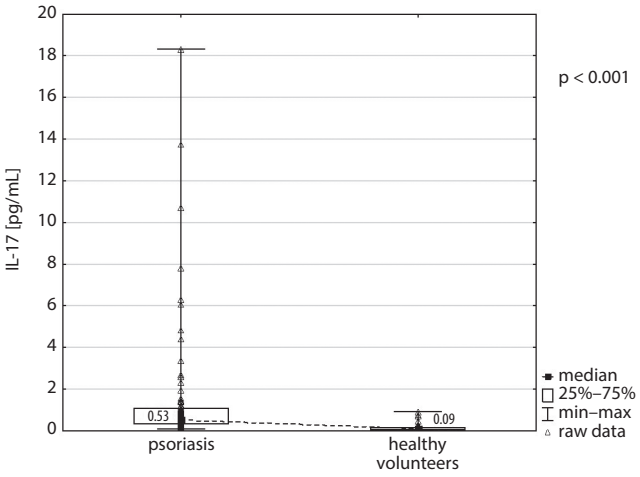

Fig. 2. Comparison of IL-17 serum concentration between psoriatic patients and healthy volunteers

Osteopontin and interleukin-17 serum concentrations vs clinical severity of psoriasis

No correlation between OPN serum concentration and psoriasis severity measured with PASI, BSA and DLQI was found. However, IL-17 serum concentration correlated positively with psoriasis severity measured with PASI, BSA and DLQI (r > 0, p < 0.05; Table 2). This means that the higher the IL-17 serum concentration the patients had, the greater the psoriasis severity they had, on average.

Interleukin 17 serum concentration significantly differed between the PGA groups of psoriasis severity in the examined patients (p = 0.027, Table 3). The examined patients

Table 1. Characteristics of psoriatic patients

Parameter	Min–Max	Median	IR
Age [years]	18–77	47	21
Age of onset [years]	0.5–65	25	18
Psoriasis duration [years]	0–48	19	20
PASI	3–45	13	10
BSA (%)	5–90	25	35
DLQI	1–29	12	9

PASI – Psoriasis Area and Severity Index; BSA – Body Surface Area; DLQI – Dermatology Life Quality Index; IR – interquartile range.

Table 2. Correlations between OPN and IL-17 serum concentrations and psoriasis severity in the patients examined

Severity of psoriasis	OPN [ng/mL]		IL-17 [pg/mL]	
	r	p-value	r	p-value
PASI	0.160	0.100	0.211	0.029
BSA [%]	0.119	0.224	0.251	0.009
DLQI	–0.053	0.589	0.299	0.002

OPN – osteopontin; IL-17 –interleukin 17; PASI – Psoriasis Area and Severity Index; BSA – Body Surface Area; DLQI – Dermatology Life Quality Index.

Table 3. Osteopontin and IL-17 serum concentrations vs PGA in the psoriatic patients

Proteins	Severity of psoriatic changes (PGA)	Parameter of proteins			Significance of differences	
		min–max	median	IR	H	p-value
OPN [ng/mL]	minimal	14.73–127.81	32.99	12.97	1.309	0.520
	mild	8.60–185.88	30.96	16.18		
	moderate	0.15–78.67	31.69	21.57		
IL-17 [pg/mL]	minimal	0.16–1.08	0.35	0.19	7.238	0.027
	mild	0.10–18.31	0.52	0.83		
	moderate	0.12–13.74	0.74	1.15		

The proteins were not analyzed in severe psoriasis according to PGA, due to the small sample size in this group (n = 2). OPN – osteopontin; IL-17 – interleukin 17; PGA – Physician Global Assessment; IR – interquartile range.

Table 4. Osteopontin and IL-17 serum concentrations vs PASI in psoriatic patients

Proteins	PASI	Parameter of proteins			Significance of differences	
		min–max	median	IR	Z	p-value
OPN [ng/mL]	below 25	0.15–127.81	31.65	17.37	0.977	0.328
	25–45	24.51–185.88	30.80	32.5		
IL-17 [pg/mL]	below 25	0.10–18.31	0.49	0.62	2.987	0.003
	25–45	0.12–6.32	1.59	2.95		

PASI below 25 (n = 95); PASI 25–45 (n = 12). OPN – osteopontin; IL-17 – interleukin 17; PASI – Psoriasis Area and Severity Index; IR – interquartile range.

with moderate severity of psoriatic changes according to PGA had the highest IL-17 serum concentration (0.74 pg/mL on average), the patients with mild changes had lower IL-17 (0.52 pg/mL on average) and the patients with minimal changes had the lowest IL-17 serum concentration (0.35 pg/mL on average). However, OPN serum concentration did not significantly differ between the PGA groups of psoriasis severity in the examined patients (p = 0.520).

Osteopontin serum concentration did not significantly differ between psoriatic patients with PASI below 25 and between 25 and 45. Interleukin 17 serum concentration was significantly higher in psoriatic patients with PASI 25–45 (1.59 pg/mL on average) than in psoriatic patients with PASI below 25 (0.49 pg/mL on average; Table 4).

Discussion

Elevated levels of OPN lead to reduction of IL-17 secretion, causing a modulation of Th17 response, which may be suggestive of the existence of a positive feedback between IL-17 and OPN. Studies in mice with model autoimmune encephalomyelitis (EAE) showed that, due to an interaction between OPN and integrin receptor β3 of CD4+ T cells, OPN is also capable of increasing IL-17 secretion directly. Moreover, anti-OPN treatment reduced the production of IL-17 and reduced the clinical symptoms of EAE.[15] Murugaiyan G. et al.[15] also evaluated OPN expression in the dendritic cells and found it was significantly higher in patients with multiple sclerosis (SM) than in healthy volunteers. In the SM patients studied, OPN secreted by the dendritic cells stimulated Th17 cells to increase production of IL-17.[15]

It has also been suggested that OPN activates the immune response of the Th1 cell subpopulation.[7] Buback et al.[7] found a strong expression of OPN in the psoriasis plaques, which confirms a possible relationship between OPN and both Th1 and Th17 response.[7] They surmised that in individuals genetically predisposed to psoriasis, OPN stimulates the antigen presenting cells, i.e., dendritic cells and Langerhans cells, in response to an injury or bacterial antigens.[7] Buommino et al.[16] found that expression of OPN was associated with an increased expression of IL-1β, INFγ and tumor necrosis factor α (TNF-α) with simultaneous low expression of IL-10 and IL-4 in psoriatic patients.[16] These results are consistent with the data showing a possible role of OPN as an early protein responsible for lymphocyte differentiation towards Th1 responses in patients with psoriasis.[16] It is known that OPN, by interacting with integrins and the CD44 molecule, strengthens the Th1 response and suppresses Th2, which is explained by lack of OPN expression in patients with atopic dermatitis.[16]

In our study, however, no significant correlation between the serum concentration of OPN and IL-17 was observed in the study subjects with psoriasis (p = 0.139) and the healthy volunteers (p = 0.501), which may have been a result of no significant correlation between the OPN concentration and the clinical severity of psoriasis in the subjects of our study. In contrast, serum IL-17 concentration was found to correlate positively with PASI, BSA, PGA, and DLQI. Thus, it is likely that the relationship between OPN

and IL-17 is complex and may be independent of psoriasis severity.

In imiquimod-induced psoriasis experimental animal models, Frenzel et al.[17] observed that increased OPN concentration may promote an inflammatory process mediated by Th17 cells, whereas the OPN-deficient mice studied presented an altered composition of inflammatory cells in the skin, lymph nodes and spleen.[17] A decrease in OPN concentration, however, was found to be associated with a decrease in the Th17 lymphocyte subpopulation in the lymph nodes and CD8+ NK cells and antigen presenting cells (APC) in the skin.[17] The findings of the Frenzel et al.[17] studies infer that OPN attracts these cells to the inflammation sites and that the absence of chemotaxis stimulated by OPN may cause a partial reduction in the influx of the immune cells.[17] The authors also observed that in mice with OPN deficiency a lack of B cell expression in the lymph nodes and spleen may further confirm the participation of OPN in the modulation of systemic inflammation.[17] This is in agreement with the results of other studies carried out on animal models in which OPN promoted polyclonal B cell activation.[18]

Chen et al.[19] observed that an increased OPN level correlated positively with the level of IL-17 in the synovium of the joints in the course of rheumatoid arthritis.[19] Similarly to IL-6 and IL-1β, OPN promotes differentiation of T cells towards Th17 cells.[19] Interestingly, anti-OPN treatment in the animal model of SM resulted in alleviation of the disease symptoms even though no change in IL-6 activity was observed.[19]

Zheng et al.[20] found a positive correlation between OPN concentration and the expression of the 2 known inflammation modulators, i.e., CD29 and CD44 receptors in patients with acute coronary syndrome.[20] They surmised that the blockage of the CD44 receptor in OPN-stimulated cells significantly reduces the differentiation of Th17 cells.[20]

In our study, a significantly higher concentration of OPN in the group of psoriatic patients in comparison to the healthy volunteers ($p < 0.001$) was observed, which is in agreement with the results reported by other authors.[21–24]

In 2016, Abdel-Mawla et al.[25] observed significantly higher OPN expression in the psoriatic plaques in comparison to the healthy skin as well as to the unchanged skin in psoriatic patients. Moreover, the expression of OPN in the unchanged skin of psoriatic patients was significantly higher than in the healthy skin of the healthy volunteers. It is worth noting that the expression of OPN in the healthy volunteers was limited to the basal layer, whereas in the unchanged skin in patients with psoriasis it was also present in the other layers of the epidermis. The authors also showed a positive correlation of OPN expression with the density of inflammatory infiltration in the skin.[25] This study confirms the participation of OPN in the local inflammatory process occurring in psoriatic lesions. It seems that an increased concentration of OPN in the unchanged skin of psoriatic patients may stimulate chemotaxis of inflammatory cells towards the predisposed site and contribute to the development of psoriatic plaque.

Amin et al.[26] also observed the presence of OPN in the vascular endothelial cells of patients with psoriasis, which could suggest its role in angiogenesis. This may be confirmed by the influence of OPN on the stimulation of vasodilatation observed by Gürsoy et al.,[11] which is associated with IL-1 activation and its effect on endothelial cells as well as the stimulation of phagocytic mononuclear cell chemotaxis.[11]

El-Eishi et al.[27] noticed that after the applied anti-psoriatic treatment, OPN expression was still significantly higher than in the healthy volunteers.[27] The greatest decrease in OPN expression was observed in patients undergoing PUVA (psoralen and ultraviolet A) therapy despite their lowest PASI reduction.[27] Conversely, the greatest PASI improvement was observed in the patients treated with ciclosporin in whom a decrease in the OPN concentration was smaller.[27]

In our study, no correlation between OPN serum concentration and clinical severity of psoriasis was found. What is more, OPN concentration did not significantly differ between the group of patients with mild psoriasis and the group with moderate or severe disease severity ($p = 0.713$). Similar results were obtained by other authors who did not find a relationship between plasma OPN concentration and PASI value[16,21,24,28–30] either. However, different data was presented by Kadry et al.,[22] who showed a positive correlation between plasma OPN concentration and the clinical severity of psoriasis. Abdel-Mawla et al.,[25] using immunohistochemistry, also observed a positive relationship between the OPN expression in the affected psoriatic skin and PASI.

In our study, there was no significant correlation between the OPN concentration and the PGA value ($p = 0.520$) as well as between the OPN concentration and the DLQI value ($p = 0.589$). Similarly, Chen et al.,[21] in their study group of 40 patients, did not observe a relationship between the OPN concentration and the PGA index either.

Conclusions

In light of recent studies, there is an undeniable link between OPN and IL-17. The aforementioned scientific studies provide evidence that increased serum concentrations of OPN and IL-17 contribute to the development of autoinflammatory and autoimmune diseases, including psoriasis.

Although our study did not reveal a correlation between the OPN and IL-17 values in the psoriatic patients studied, it should be emphasized that their concentrations were significantly higher in comparison to the healthy volunteers, which is undoubtedly a signal of some ongoing pathological process. Yet, this issue still awaits further research.

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The effects of alternate irrigation of root canals with chelating agents and sodium hypochlorite on the effectiveness of smear layer removal

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Abstract

Background. After the mechanical preparation of a root canal, the canal walls are covered with a smear layer. In order to deeply clean the dentinal tubules, removal of the smear layer is recommended. There is no consensus on the length of time of rinsing with chelating agents or irrigation with alternating chelating agents and sodium hypochlorite (NaOCl).

Objectives. The aim of the study was to evaluate the effectiveness of smear layer removal using 4 irrigation protocols.

Material and methods. We prepared 42 straight root canals to size ISO40/04 and assigned them into 4 study groups (n = 10) and a control group (n = 2). The root canals were irrigated as follows: in the control group, 180 s with 5.25% NaOCl; in group 1, 60 s with 40% citric acid (CA) and 120 s with NaOCl; in group 2, 120 s with CA and 120 s with NaOCl; in group 3, 30 s CA, 30 s with NaOCl, 30 s CA and 120 s with NaOCl; and in group 4, 60 s with CA, 30 s with NaOCl, 60 s with CA, and 120 s with NaOCl. The roots were split longitudinally and the root canals were observed under ×200–500 magnification. The root canal walls were analyzed in areas 2 mm, 6 mm and 10 mm from the apex.

Results. In the apical and medial sections, the best effects were achieved in groups 3 and 4. In coronal sections, no significant differences between experimental groups were found.

Conclusions. Within the limitations of this study, it can be concluded that irrigation with alternating NaOCl and CA was the most effective at smear layer removal, regardless of the irrigation time.

Key words: irrigation, citric acid, sodium hypochlorite, smear layer, root canals

Introduction

The effects of endodontic treatment depend mainly on decontamination and complete obturation of the root system of the teeth.[1–10] During the mechanical preparation of root canals, a smear layer is created.[1–7] Its role in endodontic treatment is still debated. The smear layer is composed of inorganic components (water and dentin debris), as well as organic components (pulp remnants, collagen, bacteria). It covers the dentin surface and clogs the dentinal tubules. It is supposed that the smear layer should be removed after the mechanical preparation of root canals, because the sealing effect of canal obturation can be marred as a result of changes in the volume of the smear layer, and because the smear layer prevents access to the dentinal tubules, where pathogens can be located.[6–8] Dissolution of the smear layer opens the dentinal tubules and increases the permeability of dentin, as a result of which antiseptic solutions and sealers can penetrate the dentinal tubules to neutralize and seal in microorganisms.[8]

Sodium hypochlorite (NaOCl; for the dissolution of organic components) and chelating agents (for the dissolution of inorganic components) are used to remove the smear layer. Commonly used chelating agents include EDTA (in the form of disodium salt of ethylenediaminetetraacetic acid) and citric acid (CA). Usually a 17% solution of EDTA and 20% or 40% solutions of CA are used; 17% EDTA has similar chelating properties to 20% CA.[9,10]

Most studies on removal of the smear layer are based on scanning electron microscopy (SEM). These tests are very expensive, and are flawed due to the method of specimen preparation.[1,2,6] Thanks to the development of optical microscopy and digital image processing, it is possible to obtain sharp microphotographs in ×500–1000 magnification without the need for physical and chemical processing of the specimens. Despite numerous studies, the exact concentration, time and sequence of application of irrigating liquids for optimal removal of the smear layer is still not known.[3,4,9] The null hypothesis for this study assumes that better effectiveness of smear layer removal will be achieved in canals irrigated with a chelating agent for a longer period. The aim of the study was a comparative analysis of the effectiveness of smear layer removal using 4 proposed irrigation protocols.

Material and methods

In the study, we used 42 human upper incisors extracted for periodontal reasons. The extracted teeth were stored in 1% solution of chloramine. The anatomical crowns were resected using drills with diamond coating under constant water-air cooling. The working length was determined using size 10 C-files (VDW GmbH, Munich, Germany) to reach the anatomical foramen, then deducting 0.5 mm from the obtained length. Root canals were prepared using Reciproc 25 and 40 instruments with a Silver Reciproc endodontic micromotor (all from VDW GmbH). The canals were then calibrated using size 40 K-files (VDW). Each tool was covered with a small amount of FileCare lubricant (VDW GmbH) before insertion into the canal, and between the cycles, the canals were irrigated with 5.25% NaOCl. After the preparation of the root canals, the root apexes were sealed with sculpting wax to avoid any overflow of liquids through the apexes. The roots were then randomly divided into 4 equal study groups (n = 10) and a control group (n = 2). The canals were irrigated according to the following protocols:

- control group:
  5.25% NaOCl – 180 s;
- group 1:
  40% CA – 60 s,
  5.25% NaOCl – 120 s;
- group 2:
  40% CA – 120 s,
  5.25% NaOCl – 120 s;
- group 3:
  40% CA – 30 s,
  5.25% NaOCl – 30 s,
  40% CA – 30 s,
  5.25% NaOCl – 120 s;
- group 4:
  40% CA – 60 s,
  5.25% NaOCl – 30 s,
  40% CA – 60 s,
  5.25% NaOCl – 120 s.

Each of the liquids listed was inserted into the canal through a beveled 0.4 × 19 mm needle, using reciprocating motion in small portions (1 mL). Each portion was activated with ultrasounds for 5 s using an ISO 35 spreader (VDW GmbH) on the E1 tip of a Smart Piezo scaler (Mectron SpA, Carasco, Italy). Each fluid exchange and activation cycle lasted 15 s; therefore, the stages lasting 30 s, 60 s and 120 s were completed in 2, 4 and 8 cycles of irrigation–activation, respectively.

Distilled water was applied in each group at the end of irrigation. After the irrigation of the root canals, the roots were incised along the axis using a separator with diamond coating on both sides under constant water and air cooling. During the incision, special attention was paid not to damage the canal walls. Then the roots were split using a chisel, obtaining 2 parts with visible canal walls.

The prepared specimens were observed using a Nikon Eclipse LV100 microscope (Nikon Corp., Tokyo, Japan) at ×200–500 magnification. The canals of both split root parts were analyzed on 3 levels: coronal (10 mm from the apex), medial (6 mm from the apex) and apical (2 mm from the apex). Each observation with a manual change of the height of the microscope platform (the distance from the lens) was aimed at determining the limit points of a sharp image of the given portion of the specimen. The determined values were then entered into

NIS-Elements Advanced Research software (Nikon Instruments Inc., Melville, USA). A computer-controlled digital camera took 30–90 pictures in 2560 × 1920 resolution while moving the specimen away from the lens in 0.5-micrometer increments within the determined limits. The aggregate images of the canal dentin surface were obtained by superimposing several dozen pictures with an aggregate resolution of 0.14 μm/pixel, whereas the computer software used an algorithm for selective superimposition of parts of images with sharp contours. The obtained images were saved to graphic files, encoded and analyzed. We modified the system described by Prado et al. to assess the effectiveness of smear layer removal[5] (Fig. 1):

1 – no smear layer;

2 – a small area covered with the smear layer, most of the tubules open;

3 – the smear layer covering most of the examined dentin surface;

4 – the smear layer covering the dentin surface completely.

Two independent observers conducted a blind analysis and assessment of encoded groups. If the specimen assessment was not unanimous, the observers reached a consensus. The collected data was saved to a database, decoded and then subjected to statistical analysis using Kruskal–Wallis and Kendall's tau tests, with the threshold of statistical significance set at $p \leq 0.05$.

Results

We did not observe 100% effective smear layer removal in any of the study groups. In the control group, all of the specimens were contaminated with smear layers. The most effective removal of the smear layer was observed in groups 3 and 4. No statistically significant differences were found between groups 3 and 4 in any of the 3 parts of the root canals ($p = 0$). The differences between groups 3 and 4 and groups 1 and 2 were statistically significant in the apical and medial sections of the root canals (apical: $p = 0.006$; medial: $p = 0.019$).

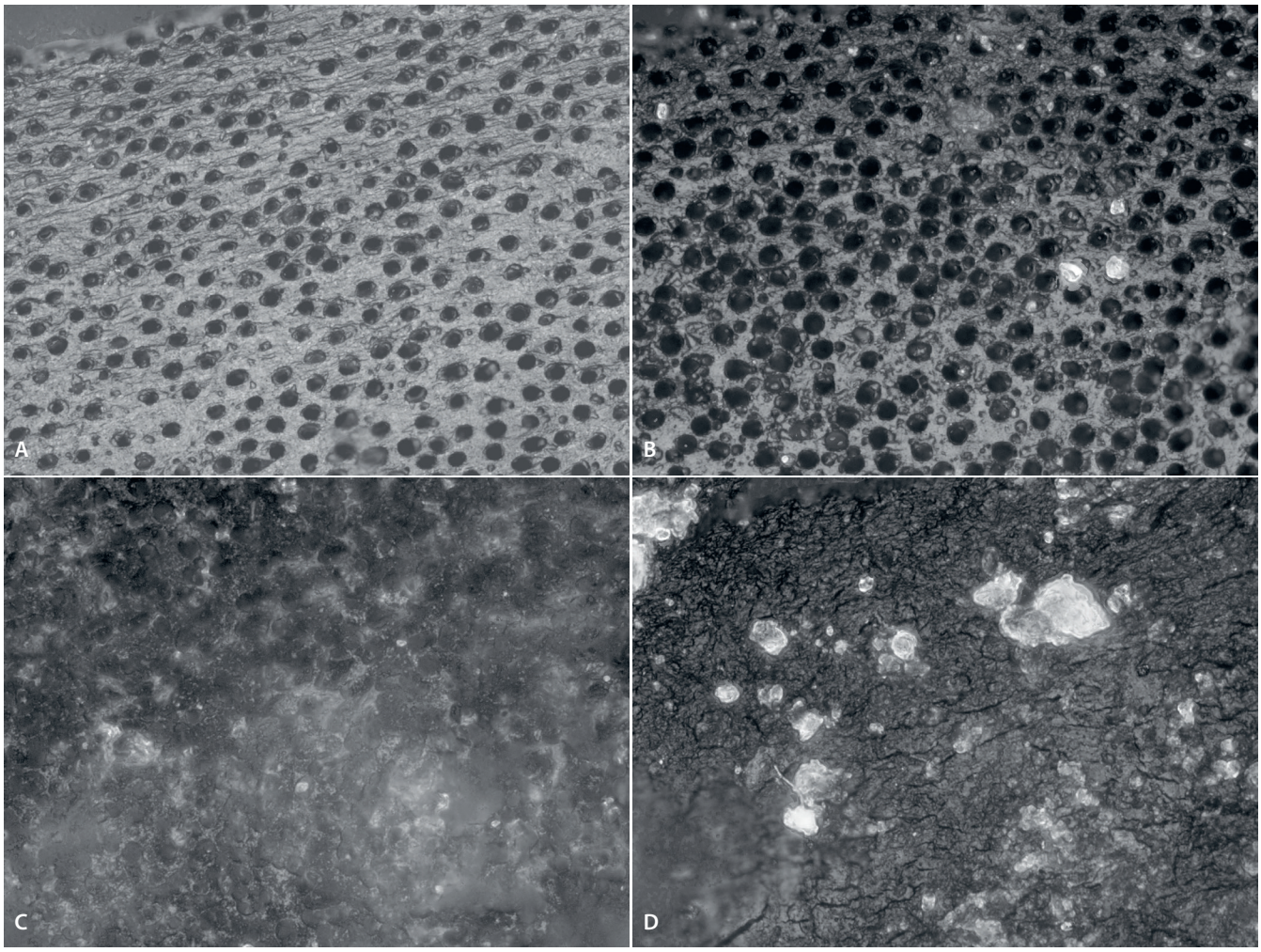

Fig. 1. Exemplary images of the scoring calculation method: A – score 1 (clean canal wall with no smear layer); B – score 2 (most of the tubules opened with small, partial area covered with smear layer); C – score 3 (most of the surface covered with smear layer with few tubules opened); D – score 4 (surface completely covered with smear layer)

Table 1. Examples of the scoring calculation method: (a) score 1: a clean canal wall with no smear layer; (b) score 2: most of the tubules opened with a small partial area covered with the smear layer; (c) score 3: most of the surface covered with the smear layer, with few tubules opened; (d) score 4: surface completely covered with the smear layer

Score	Apical				Middle				Coronal			
	group 1	group 2	group 3	group 4	group 1	group 2	group 3	group 4	group 1	group 2	group 3	group 4
Score 1	8	10	16	16	11	12	17	18	14	15	18	18
Score 2	8	7	4	4	6	6	3	2	4	4	2	2
Score 3	4	3	0	0	3	2	0	0	2	1	0	0
Score 4	0	0	0	0	0	0	0	0	0	0	0	0

However, in the coronal part, no statistically significant differences were found (p = 0.22). The data is presented in Table 1.

Discussion

Examination using SEM is most commonly used to assess the effectiveness of smear layer removal and to observe the dentin surface. While preparing specimens for such tests, it is necessary to dehydrate and macerate the specimen in alcohol in increasing concentrations (observation in high vacuum conditions) and apply a nanolayer of metal. As a result of these processes, the specimen is damaged permanently, as all the organic structures become denaturated and collapse due to dehydration. Another disadvantage of SEM is the fact that it is impossible to use the specimen again for further tests. Therefore, assessing the exposed matrix of collagen fibers after chelating agents is impossible in a standard SEM test.[1,2,6] Tay et al. used an additional stage of impregnating specimens in silazane before applying the metal, which allowed different images to be obtained than the ones observed in standard SEM.[6] It is likely that SEM observations of dentin after the use of chelating agents did not constitute a complete reflection of the dentin surface. Collapsed organic structures, such as exposed collagen, are coated with a nanolayer of metal; therefore, it is not an actual image of the dentin after demineralization.[6] Exposed collagen fibers are not hybridized by sealers and can undergo bacterial and enzymatic degradation.[7] The conclusion is that NaOCl should be used after chelating agents to dissolve the exposed protein matrix and ensure penetration into open dentinal tubules.[7,10–12] Therefore, in this study, each irrigation protocol was completed with NaOCl.

Due to the numerous flaws of SEM, attempts were made to take different images of dentin. De-Deus et al. suggested the use of a computer-controlled optical microscope as an alternative to SEM. In comparison with SEM, examination with an optical microscope is possible without any additional chemical processing of the specimens.[2–4] In their studies, De-Deus et al. observed the speed of smear layer removal and dentin demineralization using various chelating agents. This study employed a similar method of obtaining images of the surface of dentin. Sharp, clear pictures can be obtained by selective superimposition of several dozen stacks taken at various distances from the specimen, at 50 μm intervals. One of the biggest advantages of this method is its low cost and low time consumption. The main limitation is its ×500–1000 magnification, which is too low to observe nanostructures. However, it is sufficient for analyzing the effectiveness of smear layer removal.

Smear layer removal is one of the important stages of root canal irrigation. Sodium hypochlorite is used to remove the organic components of the smear layer, while the inorganic components are removed using chelating agents of various strengths and concentrations. To this day, there is no consensus regarding the best sequence, concentration and time of canal irrigation with various liquids.[1–5,9] The most commonly used chelating agents are EDTA and CA. De-Deus et al. analyzed peracetic acid and etidronic acid (HEBP) compared to EDTA.[3,4] Their studies showed that HEBP is a much weaker chelating agent than EDTA, but that a 2.25% concentration of peracetic acid can be used as an alternative to 17% EDTA. Our study employed 40% CA, which is a very strong chelating agent. The recommended time of root canal irrigation with 17% EDTA is 120–180 s; for 40% CA, the time can be shorter due to the higher reactivity of the acid. This study used 4 irrigation protocols. The aggregate irrigation time with CA was 60 s (groups 1 and 3) or 120 s (groups 2 and 4). In groups 3 and 4, the canals were irrigated with CA in 2 cycles, 30 s and 60 s, respectively. Between the chelating agent irrigation cycles, the canals were irrigated with 5.25% NaOCl in order to dissolve exposed organic substances (organic components of the smear layer, exposed collagen). During the alternate irrigation, interactions between the agents used must be taken into consideration. In the case of irrigation with alternating NaOCl and chelating agents, larger volumes of liquids should be used, as the infused liquid becomes inactivated due to chemical reactions with the other liquid in the canal.

This study took these interactions into account and used short infusions with short ultrasound activation in order to ensure the best distribution of the liquids in the endodontic system. A study by Karunakaran et al. showed that ultrasound activation increases the efficiency and effectiveness of root canal irrigation. Thanks to alternating acoustic waves and vibrations, the liquids penetrate the canal grooves and irregularities.[11] A very large number of studies concerning the effectiveness of smear layer removal did not employ

ultrasound activation.[1–6,9] Therefore, it is difficult to relate those studies to clinical situations, as ultrasound activation is a standard procedure in modern endodontics. In most studies, the least effective smear layer removal was obtained in the apical section.[5,9,12–15] In this study, we obtained a very high degree of smear layer removal in the apical section in groups 3 and 4, probably due to ultrasound activation and alternating application of the chelating agent and NaOCl. It is interesting that there were no differences between groups 3 and 4 despite the fact that the aggregate irrigation time with CA was 60 s in group 3 and 120 s in group 4.

In this situation, we rejected the null hypothesis as the study showed that smear layer removal is facilitated not by the duration of irrigation with a chelating agent, but rather by alternate irrigation with hypochlorite. Irrigation with hypochlorite probably increases the effectiveness of the subsequent CA irrigation cycle. This may be due to exposure of the organic structures in the deeper parts of the smear layer and the dentin (collagen) during the 1st irrigation cycle with the chelating agent. Irrigation with NaOCl dissolves organic substances, ensuring a better reaction between the subsequent application of the chelating agent and the canal wall. This explains the statistically significant differences between group 2 (irrigation with the chelating agent in a single 120-second cycle) and group 3 (irrigation with the chelating agent in 2 30-second cycles). The shorter duration of irrigation with the chelating agent was somewhat compensated by better penetration of the 2nd cycle of irrigation with the chelating agent, following hypochlorite. This phenomenon requires further studies employing other methods to fully understand the nature of changes in the morphology of the surface of dentin caused by irrigation.

To sum up this study (and its limitations), we can state that irrigation with 40% CA alternating with 5.25% NaOCl was the most effective in removing the smear layer.

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Microbiological, antioxidant and lipoxygenase-1 inhibitory activities of fruit extracts of chosen *Rosaceae* family species

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Conflict of interest
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Abstract

Background. Extracts from the *Rosaceae* family fruits are rich in natural, biologically active polyphenols, but their antibacterial properties are still poorly understood. Therefore, we focused our research on their activity against uropathogenic *Escherichia coli* strains. This research also concerned the proof of their ability to reduce oxidative stress and modulate the activity of lipoxygenase-1 (LOX-1). It is well-known that plants represent a source of bioactive compounds whose antioxidant activity may be useful in protecting against oxidative damage in cells, which have been linked to the pathogenesis of many oxidative diseases.

Objectives. The study determined the biological activity of methanol (ME) and water (WE) extracts rich in polyphenols from the hawthorn (*Crataegus monogyna* Jacq.), dog rose (*Rosa canina* L.), quince (*Cydonia oblonga* Mill.), and Japanese quince (*Chaenomeles speciosa* (Sweet) Nakai).

Material and methods. The antioxidant capacity was evaluated using 1,1diphenyl-2-picrylhydrazyl (DPPH$^{\bullet}$) and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS$^{+\bullet}$) radical scavenging methods. The inhibition of liposome membrane oxidation was studied using the thiobarbituric acid reactive substances assay. Lipoxygenase-1 inhibitory activity was measured using the spectrophotometric method. Bacterial growth was determined by evaluating the number of colony forming units per milliliter (CFU/mL). Hydrophobicity was established with salt aggregation hydrophobicity test (SAT). Swimming and swarming motilities were evaluated using soft-agar plates. Production of curli fimbriae was estimated on CFA agar. The P fimbriae were detected using the hemagglutination of erythrocytes. Adhesion of bacteria to human uroepithelial cells was assessed. The amount of biofilm was determined spectrophotometrically.

Results. We showed that most of these extracts are effective antioxidants and free radical scavengers, possess reasonable potential anti-inflammatory activity, reduce the adhesion of *E. coli* to uroepithelial cells, and reduce the ability of these bacteria to form biofilm.

Conclusions. The extracts examined, showing very promising biological properties, seem to be able to join the list of substances that can be used as dietary supplements aimed at preventing, for example, urinary tract infections, or as support of drug treatment in many diseases.

Key words: *Rosaceae* fruit extracts, antimicrobial, antioxidant, lipoxygenase-1 inhibition

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Introduction

In recent years, dozens of scientific articles containing the keywords "cranberry" and "proanthocyanidins" have been published.[1] This is a consequence of the commonly known fact that many berries (not only cranberries) contain relatively large amounts of anthocyanins, ellagitannins and proanthocyanidins.[2,3] This, combined with the other well-accepted facts that cranberry juice or extracts possess antibacterial properties, especially that they can reduce the risk of urinary tract infections, might result in the conclusion that the presence of anthocyanins and/or proanthocyanidins in certain plants is essential for their biological and/or antioxidant activity.[4,5] To test if this conclusion is true, we have chosen for the present study the methanol (ME) and water (WE) extracts of 4 fruits of plants belonging to the *Rosaceae* family and (according to the preliminary chemical analysis) possessing almost none or relatively small amounts of anthocyanins/proanthocyanidins (when compared to other bioactive phenolics). The *Rosaceae* family was chosen because many its members have been used in folk medicine. For example, rose hips (in particular those of the dog rose) were traditionally used as anti-infectious and anti-inflammatory agents.[6] Several North American First Nations have used hawthorn as a remedy against gastrointestinal disorders and respiratory problems like coughs, flu, bronchitis, and asthma, while in Chinese traditional medicine, it was used against circulatory problems, indigestion, diarrhea, and hypertension.[7] On the other hand, quince was used in Italy in folk medicine for treatment of various skin diseases and in Portugal the sedative, antipyretic, antidiarrheal, and antitussive properties of quince leaves were utilized.[8] In the current paper, the chemical analysis of the composition of the extract has been followed by an assessment of their antioxidant and lipoxygenase-1 (LOX-1) inhibitory (as a potential anti-inflammatory) properties and antibacterial effects exerted against *Escherichia coli* rods.

Material and methods

Plants and extract preparation

The raw material to study was hawthorn, dog rose, quince, and Japanese quince (J. quince). Fruits of hawthorn, dog rose and J. quince were collected in Szczytnicki Garden in Wrocław, Poland, whereas the fruits of quince were collected in the Arboretum and Institute of Physiography in Bolestraszyce, Poland. The WE and ME were prepared exactly as previously described by Sroka et al.[9]

Determination of total phenol content and identification of components

Total polyphenols were determined using the Folin–Ciocalteu method. The results were calculated as the equivalent of gallic acid (in micromoles) per gram of dry matter of the extract (μM GAE/g d.m.).

Preparation of liposomes and induction of lipid peroxidation

The method of lipid peroxidation assessment was described by Strugała et al.[10] The lipid peroxidation level in the liposomes was measured as the concentration of a thiobarbituric acid reactive substance (TBARS). The antioxidant activity of the extracts tested was expressed with the parameters: IC_{50}^{PC} (Inhibition Concentration) [mg/mL] and TEAA (Trolox equivalent antioxidant activity expressed in micrograms of Trolox per gram of dry matter of the extract – μM TE/g d.m.). IC_{50}^{PC} represented the amount of an antioxidant which causes 50% inhibition of phosphatidylcholine (PC) liposome peroxidation.

Free-radical scavenging assay

The free-radical scavenging activity of 1,1diphenyl-2-picrylhydrazyl (DPPH•) was measured according to the method described by us in an earlier work.[10] The free-radical scavenging activity of cation-radicals (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt ($ABTS^{+\bullet}$)) was assessed according to the method described by Re et al.[11] The results are expressed as μM TE/g d.m. by reference to a standard curve.

Evaluation of LOX-1 inhibition

The inhibition of soybean lipoxygenase (Sigma-Aldrich, St. Louis, USA) by fruit extracts was tested using the procedure described by Axelrod et al.[12] with modifications. The WE and ME of the plants at a concentration of 150 μg/mL were used. For comparative purposes, the non-steroidal anti-inflammatory agent Ibuprofen was used as a reference substance at a concentration of 0.75 μg/mL. Reactions were carried out in 10 mm path-length quartz cuvettes containing, in a final volume of 2.6 mL: borate buffer pH 9.0, LOX-1 (0.1 mg/mL), the extracts tested, and 50 μM linoleic acid. This mixture was incubated for 3 min at room temperature, prior to the measurement. Reference cuvettes (of 2.6 mL volume) contained sodium linoleate borate buffer and an appropriate volume of the solvent extract tested. Inhibition of LOX-1 activity was assessed through spectrophotometric monitoring of the absorbance increase at 234 nm (1 min, 2 min and 3 min after linoleic acid addition) due to formation of conjugated diene hydroperoxides during the enzymatic oxidative processes. Percentage inhibitory effect was calculated using the following formula:

$$\%inhibition = \frac{\Delta A_{control} - \Delta A_{sample}}{\Delta A_{control}} \cdot 100\%$$

where: $\Delta A_{control}$ and ΔA_{sample} denote the increase of absorbance after 3 min from substrate addition to the probe without or with the extract tested, respectively.

Bacterial strain and growth conditions

A clinical *E. coli* strain isolated from the urine of a patient hospitalized in the University Hospital in Wrocław was used. The *E. coli* stock culture was kept in the refrigerator (–40°C) on a nutrient agar plate. Before each experiment, the strain was first allowed to reach room temperature, transferred to tryptic soy agar, and incubated at 37°C for 18 h. Then, bacterial cells were incubated in tryptic soy broth with varying concentrations (1, 5, 10, 20, 30, 40, 50, 60, 65, 70, 75, and 80 mg/mL) of ME or WE of the plants tested for 24 h at 37°C. Control cultures were incubated in the absence of plant extracts.

Antimicrobial activity

The antibacterial activities of the tested fruit ME and WE were determined with the standard dilution method. After incubation with different concentrations of fruit ME and WE, the bacterial cells were centrifuged, washed 3 times in phosphate buffered saline (PBS), diluted, and cultured on nutrient agar plates for 18 h at 37°C. The effect of the tested extracts on bacterial growth was evaluated on the basis of the number of colony forming units per milliliter (CFU/mL).

Swimming and swarming motility

Swimming and swarming motilities were evaluated using soft-agar plates according to the procedures described by Hidalgo et al.[13] and Sanchez-Torres et al.[14]

Hydrophobicity of bacterial cells

Bacterial cell hydrophobicity was assessed with the salt aggregation hydrophobicity test (SAT) as described by Siegfried et al.[15]

The ability to produce curli and P fimbriae

Production of curli fimbriae was estimated by bacterial growth on CFA agar supplemented with Congo red dye as described by Rosser et al.[16] *Escherichia coli* colonies expressing curli fimbriae are able to bind Congo red dye, so they demonstrate the red color. The P fimbriae expression was detected using the hemagglutination of 3% human erythrocytes (blood group 0) in the presence and absence of 3% (w/v) D-mannose (mannose-resistant hemagglutination), as described by Latham et al.[17]

Adhesion of bacteria to human uroepithelial cells

The cell adhesion assay was performed essentially as described previously.[18]

Biofilm formation assay

The biofilm formation assay was performed according to O'Toole and Kolter.[19] After 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 days of incubation, the OD_{590} values of the stained biofilms were read using a microtiter plate reader. On the basis of the recorded optical densities (ODs) of bacterial biofilms, *E. coli* strains were divided into 4 categories: OD ≤ ODc (no biofilm producer); ODc < OD ≤ 2 × ODc (weak biofilm producer); 2 × ODc < OD ≤ 4 × ODc (moderate biofilm producer); and 4 × ODc < OD (strong biofilm producer).[20] The cut-off OD (ODc) was defined as 4 standard deviations (SD) above the mean OD of the negative control. In our study, the ODc value was 0.003.

Statistical analysis

The results of all the experiments are given as a mean value ±SD of 3 independent experiments. The data concerning the antioxidant and anti-inflammatory properties was compared using Duncan's multiple range test and analyzed using one-way analysis of variance (ANOVA). The differences in swimming and swarming motilities, bacterial adhesion, and biofilm formation by bacteria exposed and unexposed to fruit extracts were analyzed with a parametric t-test for independent samples using STATISTICA v. 9.0 (StatSoft, Inc., Tulsa, USA). P-values <0.05 were considered to be statistically significant.

Results

Total polyphenol content and extract composition analysis

The results of the total polyphenol content analysis using the Folin–Ciocalteu method of the studied extracts are presented in Table 1. Qualitative and quantitative analysis of the components present in the extracts was performed with the high-performance liquid chromatography (HPLC), and the results are presented in Table 1. As can be seen, individual extracts differed in their composition depending on the method of extraction as well as on the type of fruit. The biggest similarities can be found in the case of flavanols and flavonols that are present in ME of dog rose, quince and J. quince. All these preparations are relatively rich in quercetin 3-*O*-galactoside and quercetin 3-*O*-glucoside. These 2 components were also found in hawthorn ME, however, in much smaller amounts than in other extracts. On the other hand, among phenolic

Table 1. Total polyphenol content (TPC) and main groups of phenolic compounds (flavanols and flavonols, phenolic acids, and their derivatives) of methanol (ME) and water (WE) extracts of plants studied

Fruit	TPC [μM GAE/g d.m.]		Flavanols and flavonols		Phenolic acids and derivatives	
	ME	WE	ME	WE	ME	WE
Hawthorn	58.8 ±1.9^{c}	23.8 ±0.7^{d}	(–)epicat (75.0%) f-3-ol dimer (12.0%) f-3-ol trimer (5.7%) q-3-gal (3.8%) q-3-glc (1.2%) q-3-rut (0.3%) n.i. (2%)	n.d.	chlor ac (45.9%) p-coum ac (21.1%) phenolic ac n.i. (13.7%) neochlor ac (8.9%) n.i. (10.4%)	neochlor ac (69.4%) p-coum ac (18.0%) phenolic ac n.i. (8.0%) n.i. (5.0%)
Dog rose	46.4 ±2.9^{d}	49.3 ±1.5^{a}	q-3-gal (35.2%) q-3-glc (64.8%)	(+)cat (86.7%) procyanidin n.i. (13.3%)	gall ac (45.3%) ell ac (28.5%) der gall ac (26.2%)	der gall ac (100.0%)
Quince	152.9 ±4.1^{b}	37.0 ±1.2^{b}	q-3-gal (57.8%) q-3-glc (15.8%) q-3-pento (15.8%) q-3-rut (9.1%) n.i. (1.5%)	q-3-gal (87.5%) q-3-rut (12.5%)	chlor ac (39.8%) phenolic ac n.i. (29.9%) n.i. (30.3%)	chlor ac (38.0%) phenolic ac n.i. (43.9%) n.i. (18.1%)
Japanese quince	216.5 ±7.2^{a}	30.9 ±1.5^{c}	q-3-gal (54.7%) q-3-glc (18.8%) n.i. (26.5%)	n.d.	chlor ac (40.1%) phenolic ac n.i. (39%) neochlor ac (14.9%) dicaffe ac (4.6%)	chlor ac (81.4%) n.i. (19.6%)

d.m. – dry matter; GAE/g d.m. – equivalent of gallic acid (in micromoles) per gram of dry matter of the extract; (–)epicat – (–)epicatechin; (+)cat – (+)catechin; chlor ac – chlorogenic acid; der gall ac – derivatives of gallic acid; dicaffe ac – dicaffeoylquinic acid; ellg ac – ellagic acid; f-3-ol dimer – flavan-3-ol dimer; f-3-ol trimer – flavan-3-ol trimer; gall ac – gallic acid; neochlor ac – neochlorogenic acid; p-coum ac – p-coumaroquinic acid; phenolic ac – phenolic acid; q-3-gal – quercetin 3-O-galactoside; q-3-glc – quercetin 3-O-glucoside; q-3-rut – quercetin 3-O-rutinoside; q-3-pento – quercetin 3-O-pentoside; n.i. – not identified; n.d. – not determined. Different uppercase letters (a–d) within the same columns indicate significant differences at $p < 0.05$ in Duncan's test.

acids, the common component for hawthorn, quince and J. quince ME, and quince and J. quince WE was chlorogenic acid, which in the case of the last extract mentioned reached the level of 81.4% contribution.

Free-radical scavenging and antioxidant properties

The results of experiments in which the antioxidant and radical scavenging properties of the extracts studied have been determined are presented in Table 2. As can be seen, both extracts of J. quince appeared to possess the strongest antioxidant properties (as determined with all tests used), and the ME of these fruits was also the most effective free radical scavenger (in both DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ tests). The scavenger activity of all extracts studied is comparable to that measured by Grace et al.[21] for cranberries; however, one has to keep in mind that the activities mentioned were calculated with respect to the fresh weight of the berries (not dry mass as in our case). The weakest antioxidant properties were recorded for both types of quince extracts whereas dog rose extracts were found to be the poorest free radical scavengers.

Effects of the extracts on LOX-1 inhibition

As it follows from Table 3, all extracts studied were able to inhibit soybean LOX to an extent comparable to the inhibition effects exerted by the 200-fold smaller

Table 2. Antioxidant (IC_{50}^{PC}), Trolox Equivalent Antioxidant Activity (TEAA) and antiradical (DPPH$^{\bullet}$, ABTS$^{\bullet+}$) activities of methanol (ME) and water (WE) extracts from fruit of the *Rosaceae* family

Fruit	IC_{50}^{PC} [mg/mL]		TEAA [μM TE/g d.m.]		DPPH$^{\bullet}$ [μM TE/g d.m.]		ABTS$^{\bullet+}$ [μM TE/g d.m.]	
	ME	WE	ME	WE	ME	WE	ME	WE
Hawthorn	0.148^{b} ±0.002	1.119^{b} ±0.005	80.90^{b} ±1.1	23.80^{c} ±0.04	234.6^{b} ±9.5	103.6^{b} ±2.5	479.6^{b} ±14.5	186.2^{c} ±2.5
Dog rose	0.160^{b} ±0.035	0.208^{c} ±0.010	78.40^{b} ±1.0	55.70^{b} ±2.70	171.1^{c} ±5.1	150.7^{a} ±3.2	370.7^{d} ±9.6	365.2^{a} ±12.2
Quince	0.183^{a} ±0.002	1.792^{a} ±0.002	65.40^{c} ±1.10	10.90^{d} ±0.50	232.8^{b} ±2.9	111.5^{b} ±4.4	390.5^{c} ±17.3	247.5^{b} ±3.7
Japanese quince	0.124^{c} ±0.040	0.089^{d} ±0.042	96.76^{a} ±3.10	134.6^{a} ±10.60	275.3^{a} ±4.4	73.4^{c} ±1.4	547.7^{a} ±35.4	162.4^{d} ±2.0

Different uppercase letters (a–d) within the same columns indicate significant differences at $p < 0.05$ in Duncan's test. IC_{50}^{PC} – the concentration of antioxidant which reduces peroxidation intensity of phosphatidylcholine liposomes about 50%.

Table 3. Lipoxygenase (LOX-1) inhibitory activity (%) of methanol (ME) and water (WE) extracts at 150 µg/mL and non-steroidal anti-inflammatory agent Ibuprofen at 0.75 µg/mL

Examined substance	% LOX-1 inhibition	
	ME	WE
Hawthorn	22.6 ±3.4^{c}	47.7 ±1.5^{a}
Dog rose	45.7 ±1.0^{a}	42.8 ±1.5^{b}
Quince	10.3 ±2.3^{d} ↑	31.9 ±2.4^{c}
Japanese quince	39.8 ±1.8^{b}	25.9 ±0.5^{d}
Ibuprofen	26.1 ±1.6	

↑ – increase of LOX-1 activity. Different uppercase letters (a–d) within the same columns indicate significant differences at $p < 0.05$ in Duncan's test.

concentration of Ibuprofen. The most effective inhibitors were dog rose ME and WE and hawthorn WE; however, other extracts (with the striking exception of quince ME) also showed reasonable levels of LOX-1 inhibition. Somewhat surprisingly, we found that quince ME exerted the opposite effect to that of other extracts and increased the LOX-1 activity by approx. 10%.

Effects of the extracts on bacterial growth

Very low concentrations of hawthorn, dog rose, quince, and J. quince ME and WE had no effect on the survival of the bacteria (Table 4). In all cases, the values of CFU/mL were comparable to controls. In this group of extracts, the best bactericidal activity was observed for J. quince (both ME and WE). The values of CFU/mL at concentrations from 20 mg/mL to 80 mg/mL were gradually decreased to approx. 10^4–10^5 bacteria per milliliter. A much weaker effect was found for extracts of dog rose and quince. Both ME and WE at concentrations ranging from 20 mg/mL to 80 mg/mL slightly reduced the number of bacterial cells compared to the control. Both WE and ME of hawthorn had no impact on the survival of bacteria. For all concentrations of ME and WE used in our study, the values of CFU/mL were comparable to the control.

Effects of the extracts on *E. coli* swimming and swarming motility

An analysis of the average motility zone diameters of *E. coli* incubated with the extracts studied shows that no effects on the *E. coli* swimming ability were exerted by either ME or WE of dog rose or quince, nor by hawthorn ME (Fig. 1). The average swimming motility zone diameters of *E. coli* rods after incubation of the cells with various concentrations of these extracts were slightly lower or comparable to the control (30.0 ±2.0 mm). Water extract of hawthorn in concentrations ranging from 40 mg/mL to 80 mg/mL reduced the swimming motility zone diameters of *E. coli* down to the value of 10.0 ±1.0 mm, while in the case of lower concentrations of this extract, no such effect was found. The average swimming motility zone diameters of *E. coli* rods after incubation of the cells with various concentrations of J. quince were lower than the control (21.0 ±2.0 mm and 17.0 ±1.0 mm for ME and WE, respectively). The swarming ability of the examined rods was strongly affected by almost all concentrations of hawthorn (both ME and WE) and dog rose ME (Fig. 2). In those cases, the average swarming zone diameter was reduced from 16.0 ±2.0 mm (control) to 7–8 mm. Such effect was also noted for higher concentrations of quince and J. quince ME, where the observed swarming motility zone diameters decreased to 6–7 mm. The average swarming motility zone diameters of *E. coli* rods after incubation of the cells with

Table 4. Effect of methanol (ME) and water (WE) extracts of hawthorn, dog rose, quince, and J. quince on *E. coli* growth

Plant extract concentration [mg/mL]	Viable cell counts [CFU/mL] [×10^9]							
	hawthorn		dog rose		quince		Japanese quince	
	ME	WE	ME	WE	ME	WE	ME	WE
Control	2.4							
1	–^{a}	–	–	–	–	–	3.2	2.2
5	–	–	–	–	–	–	2.2	1.9
10	2.5	2.3	1.3	1.2	1.6	1.2	–	–
20	1.7	1.8	1.1	0.67	0.68	1.2	0.089	0.027
30	1.6	1.8	1.0	0.67	0.66	0.73	0.086	0.0076
40	1.6	1.7	0.76	0.34	0.59	0.70	0.0023	0.0044
50	1.5	1.6	0.62	0.2	0.57	0.52	0.00021	0.0012
60	1.5	1.5	0.51	0.078	0.51	0.50	0.00021	0.00068
65	1.4	1.2	0.47	0.073	0.35	0.49	0.000033	0.00048
70	1.4	1.2	0.36	0.066	0.35	0.21	0.000031	0.00032
75	1.3	1.1	0.34	0.026	0.30	0.20	0.000019	–
80	1.3	1.1	0.25	0.020	0.21	0.14	0.000014	–

[a] not tested; CFU – colony forming units.

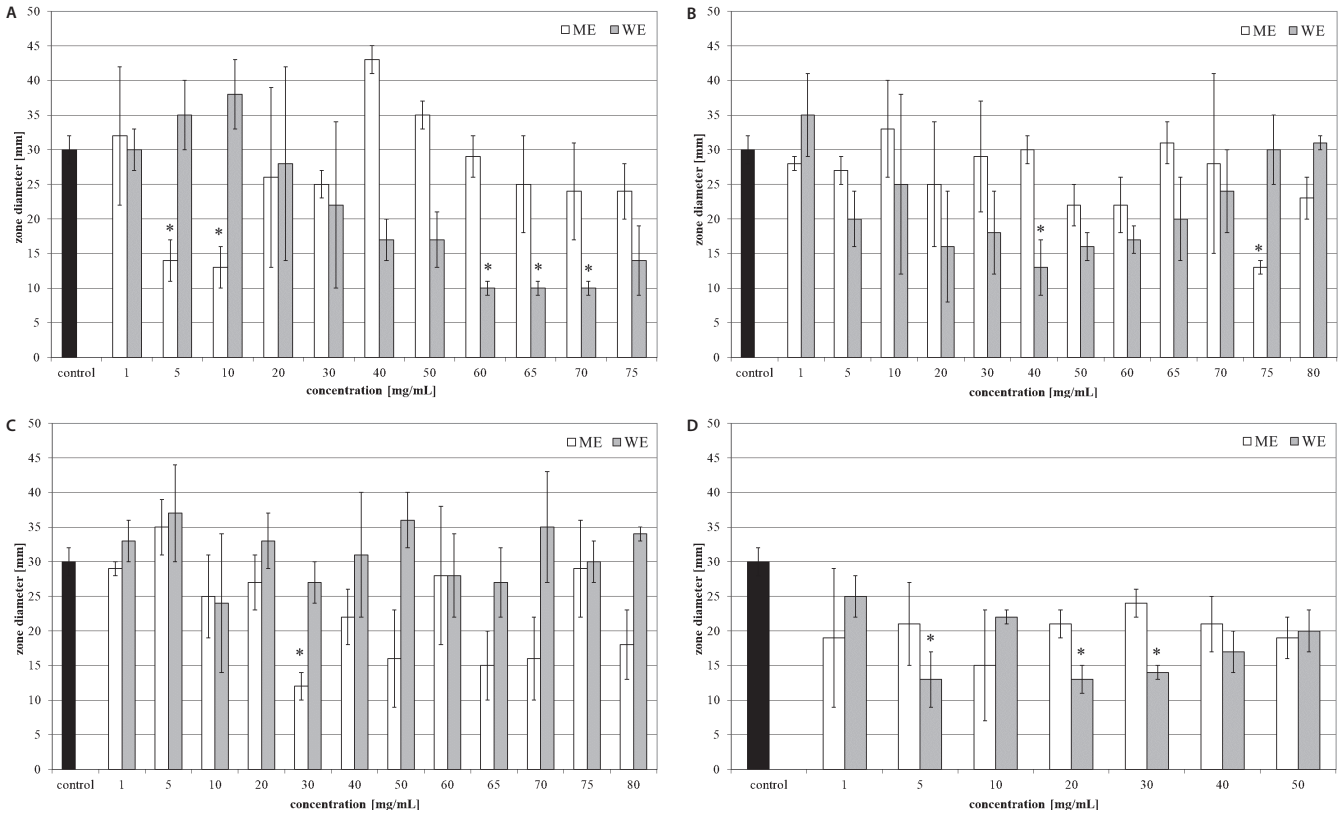

Fig. 1. Effect of methanol (ME) and water (WE) extracts of hawthorn (A), dog rose (B), quince (C), and J. quince (D) on swimming motility of *E. coli* strain; * $p < 0.05$

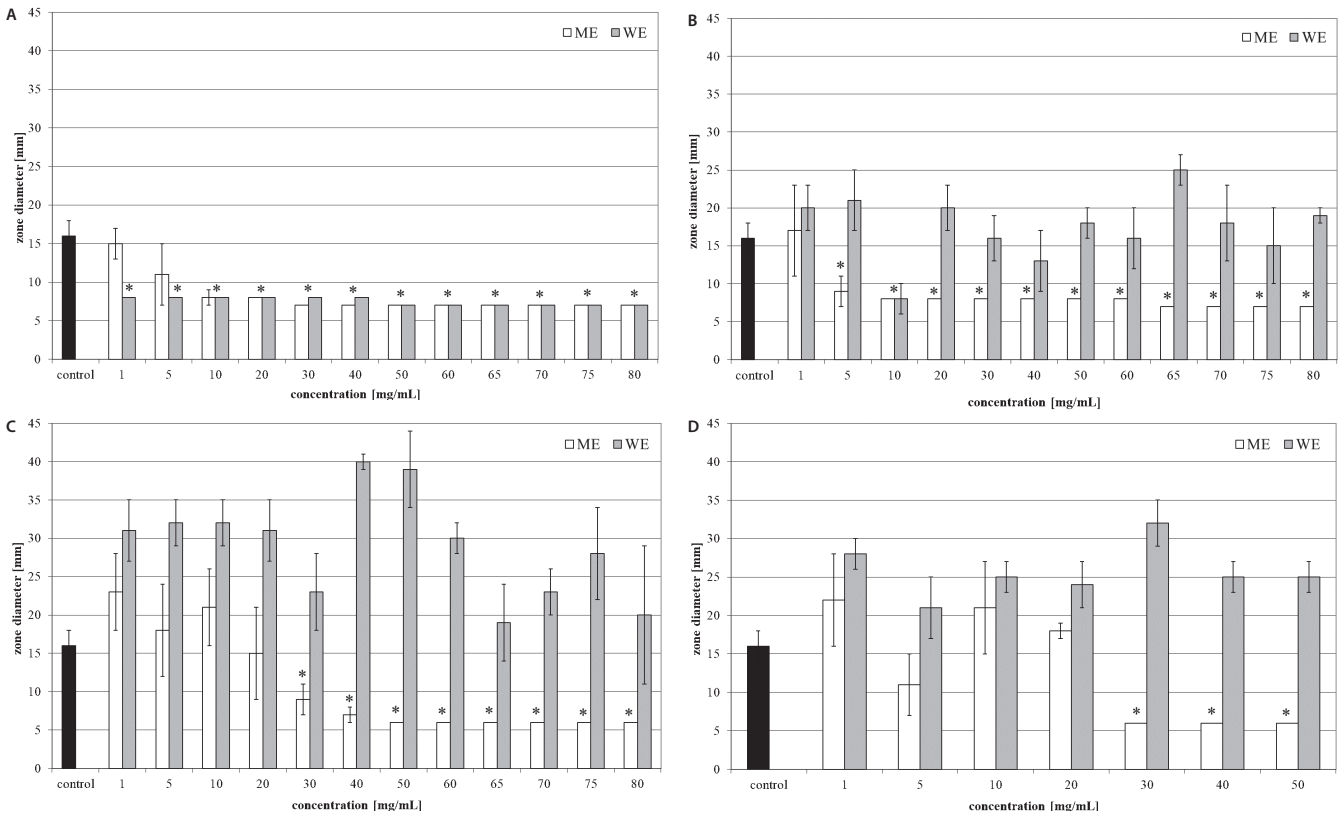

Fig. 2. Effect of methanol (ME) and water (WE) extracts of hawthorn (A), dog rose (B), quince (C), and J. quince (D) on swarming motility of *E. coli* strain; * $p < 0.05$

various concentrations of quince and J. quince WE were higher than in the control (25.0 ±2.0 mm). A similar effect on the swarming ability of the *E. coli* rods examined was also shown by dog rose WE. The average swarming motility zone diameters were slightly higher or comparable to the control (20.0 ±2.0 mm).

Effects of the extracts on the hydrophobicity of bacterial cells and on the ability to produce curli and P fimbriae

We observed that none of the tested plant extracts affected the hydrophobic properties of the bacterial cell surface. We also noted that the ability of *E. coli* to produce curli and P fimbriae was not altered by incubation of these bacteria with all tested plant extracts.

Effects of the extracts on the adhesion of bacteria to human uroepithelial cells

The dependencies of the percentage of adhesion of bacterial cells on fruit extract concentration are shown in Fig. 3 for hawthorn, dog rose, quince, and J. quince, respectively. This figure shows that there were almost no differences in the efficacy of ME and WE; however, certain fruit extracts differed in their activity. Out of the 4 extracts studied herein, only hawthorn extract showed poor effects and weakly reduced adhesion of bacteria to epithelial cells (Fig. 3A), even at high concentrations (70–80 mg/mL). Much stronger effects were recorded for dog rose and quince extracts (Fig. 3B), which were able to reduce percentage of adhesion to less than 10% when used at high concentrations. The strongest effects were found for J. quince (Fig. 3D), for which a significant decrease of adhesion was recorded for concentrations exceeding 20 mg/mL.

Effects of the extract on biofilm formation

The control samples of *E. coli* incubated up to 10 days in the absence of the fruit extracts studied herein have shown that the amounts of biofilm formed by these bacteria changed in time, reaching maxima on approx. the 3rd and 8th day of incubation (see the control bars in any panel of Fig. 4). Incubation of the bacteria with the addition of the fruit extracts studied affected the growth of biofilm within the region of the 1st and/or 2nd maximum, depending on the type of extract. The strongest reduction of biofilm was observed when the bacteria were incubated with WE of J. quince (Fig. 4D – note that J. quince was used at half of the concentrations used for other extracts) and quince, for which complete disappearance of the biofilm was observed during the whole time of observation. The ME of J. quince also showed strong biofilm reducing properties; however, the presence of some biofilm remains

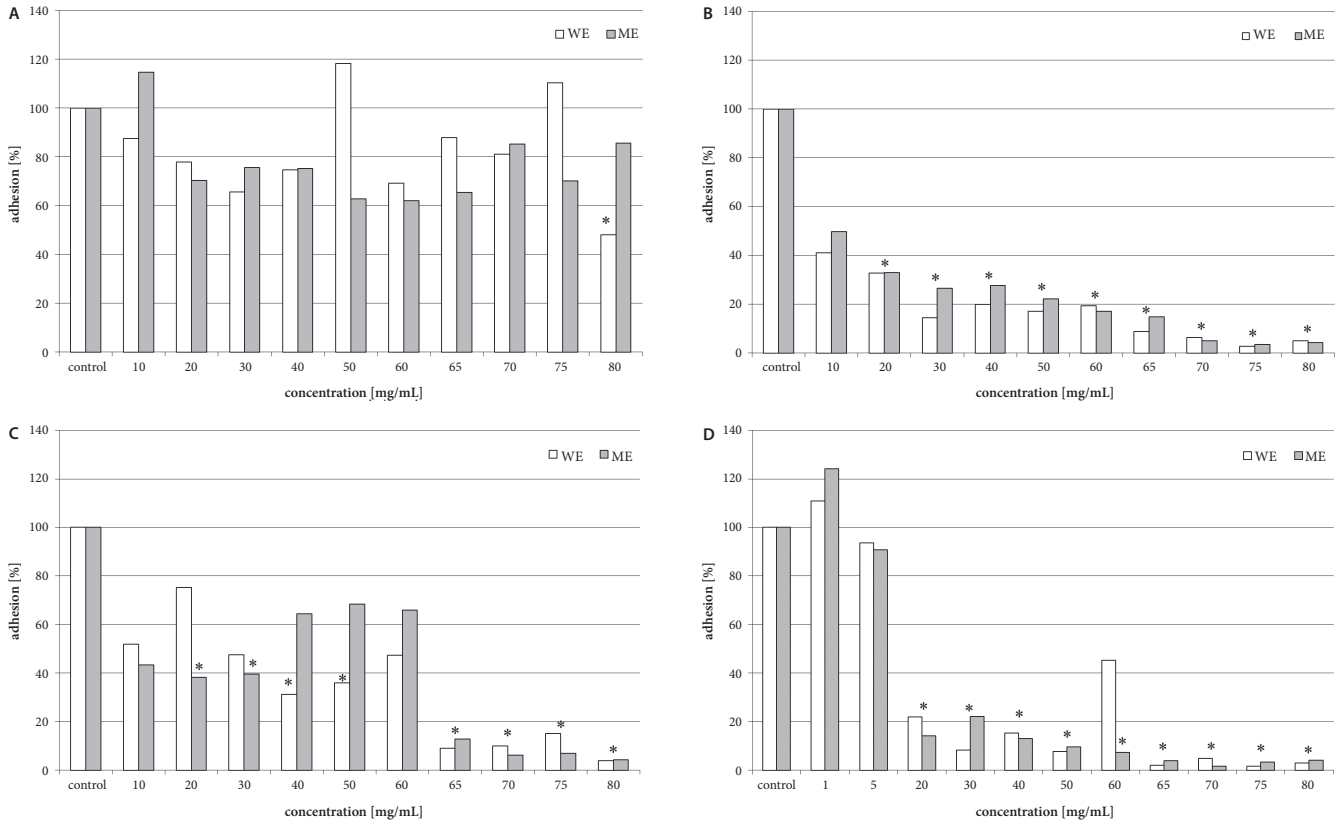

Fig. 3. Effect of water (WE) and methanol (ME) extracts of hawthorn (A), dog rose (B), quince (C), and J. quince (D) on adhesion of *E. coli* to human epithelial cells; * $p < 0.05$

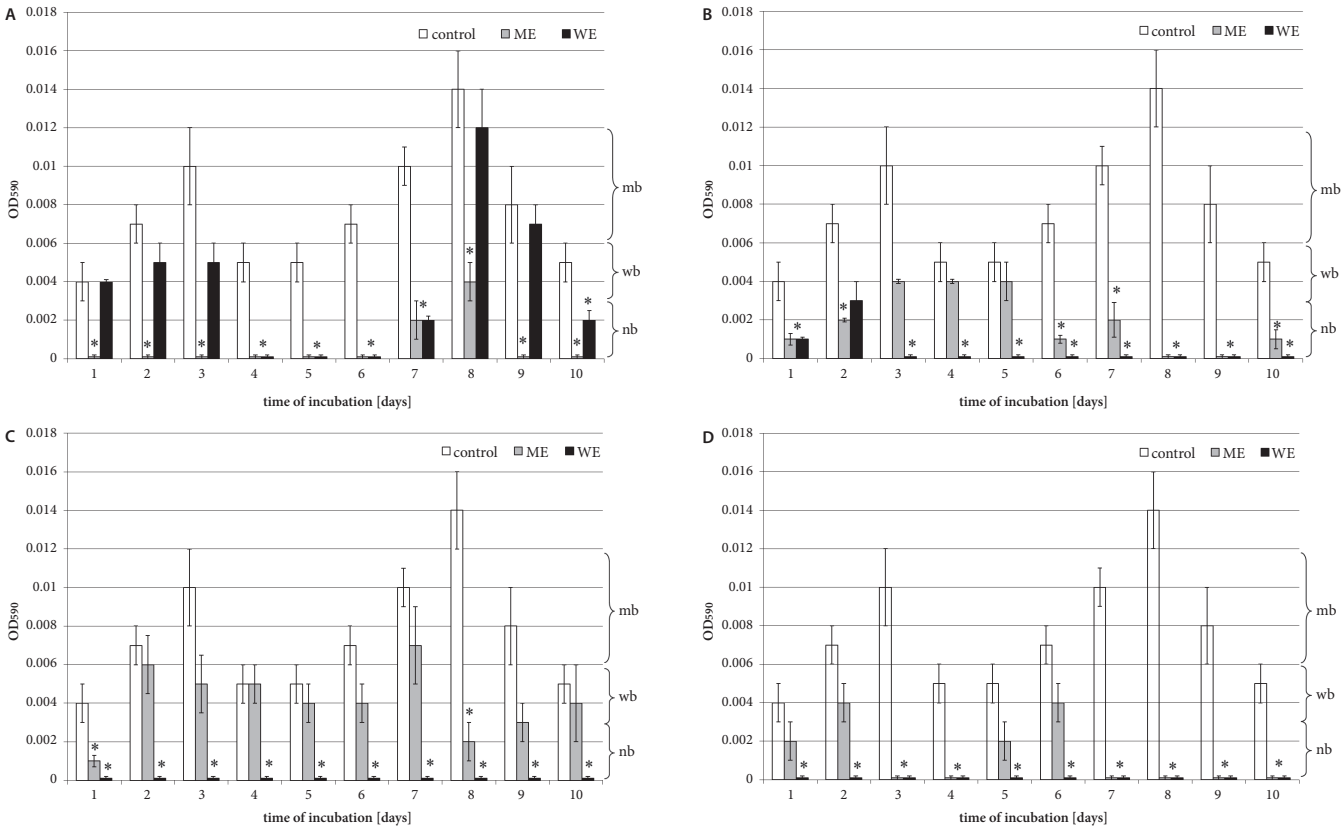

Fig. 4. Effect of water (WE) and methanol (ME) extracts of hawthorn (A), dog rose (B), quince (C), and J. quince (D) on biofilm formation ability of *E. coli* rods.

Abbreviations used for biofilm classification: nb – no biofilm; wb – weak biofilm; mb – moderate biofilm. Bars exceeding mb are classified as strong biofilm; * $p < 0.05$.

(classified as a weak biofilm) were recorded on the 2nd and 6th day of incubation. Effects comparable to those described above were also observed for hawthorn ME and dog rose WE (Fig. 4A,B). The weakest effect on the biofilm formation ability was exerted by quince ME (Fig. 3C), and the effects of dog rose ME can also be described as moderate.

Discussion

In recent years, much attention has been focused on plants containing various bioactive compounds which may demonstrate some pharmacological properties.[22] In one aspect of our research, we used a model lipid membrane (liposomes that are a spherical bilayer) as a matrix of biological membranes. Membranes are the first target of attack by exogenous free radicals at the cellular level. Peroxidation processes of membrane lipids result in disruption of their function, which is the basis of serious diseases, e.g., atherosclerosis or diabetes. Another important aspect of the research was to check whether natural plant extracts can reduce the risk of urinary tract infections. We determined the phenolic content, antioxidant activity and potential anti-inflammatory properties of 4 plant WE and ME on one hand and assessed the effects exerted by those extracts on *E. coli* on the other hand.

The research presented herein has shown that extracts from the *Rosaceae* family species are rich in phenolic compounds. Quantitative/qualitative identification using HPLC showed that the components of individual extracts belong to 3 groups of phenolic compounds: flavanols, flavonols and phenolic acids. These 3 groups of phenolic compounds in hawthorn extracts, dog rose quince and J. quince were also identified by other researchers.[23–26] The richest in phenolic compounds were the ME of J. quince (216.5 ±7.2 μM GAE/g d.m.) and quince (152.9 ±4.1 μM GAE/g d.m.). Chlorogenic acid was identified in all examined extracts.

Comparing the properties of the 2 types of extracts (ME vs WE), one can conclude that in most cases, ME are more effective as antioxidants as well as free radical scavengers. Statistical analysis of the abovedescribed data showed that there is no correlation between the total polyphenol content (TPC) and antioxidant properties (measured as IC_{50}^{PC}) of the extracts studied (correlation coefficients were –0.338 and –0.273 for ME and WE, respectively). Simultaneously, some correlations were found between the TPC and radical scavenging properties of the extracts: correlation coefficients were 0.817 and 0.782 (in DPPH$^{\bullet}$ assay, ME and WE, respectively) and 0.577 and 0.927 (in ABTS$^{\bullet+}$ assay, ME and WE, respectively). Bearing in mind the above statistical relations and comparing the results of the chemical analysis, it is hard to point to any strict correlation

between the composition of the extracts and their antioxidant/protective action in relation to oxidized lipid membranes. However, we can firmly state that the ME extract of J. quince was at the top of the tables (both phenolic content and antioxidant activity). The antioxidant and antiradical activity of J. quince has been confirmed using different methods in current literature.[27] Until now, to our knowledge, there are no reports related to the inhibition of the peroxidation of lipid systems mimicking the bilayer of biological membranes. Some reviews have been published that contain a wide range of information on both *Chaenomeles* composition and therapeutic properties.[28,29] It is noteworthy that despite the relatively low but comparable TPC content of WE and ME of the dog rose, they demonstrate a high ability to inhibit lipid peroxidation and LOX-1 activity. Dog rose is also good scavenger against DPPH and ABTS mimic radicals. In mammalian cells, LOX plays a key role in the biosynthesis of a variety of bioregulatory compounds such as hydroxyeicosatetraenoic acid, leukotrienes, lipoxins, and hepoxylines. Antioxidants interact non-specifically with LOX by scavenging radical intermediates and/or reducing the active heme site.[30] For example, in the work of Chen et al., it was shown that extract from *Prunus campanulata* Maxim, a member of the family *Rosaceae*, at concentrations of 25 μg/mL, 50 μg/mL and 100 μg/mL causes inhibitory activity of 15-lipoxygenase (15-LOX).[31] Tumbas et al. suggest antioxidant properties of wild rose tea (as detected with DPPH$^{\bullet}$ test), presumably related to the content of vitamin C and flavonoid compounds. Simultaneously, the anti-proliferative properties of this tea found in several types of human cancer cells are suggested to result from the interaction with polyphenols.[32] The review of Patel in various aspects sums up the research on the biological activity of fruits of the rose hip, and it indicates the research on the mechanisms of its pharmacological action.[33]

Anthocyanins/proanthocyanidins are often recognized as compounds that play a major role in the antibacterial activities of different fruit juices or extracts.[1,4,34] Despite this, the results presented above demonstrate that many potentially useful properties can also be present in the extracts of fruits that possess a low level or none of such components. Since in our experiments we used the uropathogenic strains of *E. coli*, it seems obvious that as the most important we consider the effects exerted on those bacteria properties which are involved in the induction of urinary tract infection. Swimming and swarming motility and adhesion to the epithelial cells as well as biofilm forming ability undoubtedly belong to these properties.[35] Analysis of the results obtained in our study leads to the conclusion that the most effective was the WE and ME of J. quince, which significantly reduced the swimming and swarming (except for WE) motility, adhesion to epithelial cells and biofilm formation ability of bacteria. Biofilm formation ability was reduced (to different extents) by all extracts studied and adhesion to the epithelial cell was reduced by all except hawthorn ME and WE. These last extracts have, however, significantly decreased the swarming motility of *E. coli*.

With the exception of hawthorn ME and WE, all extracts also showed some antibacterial properties, however, due to the moderate effect, it was not possible to determine the minimal inhibitory concentration (MIC) in the range of the extract concentrations used in our experiments. The antibacterial activity of these extracts was somehow smaller than that of cranberry extract Żuravit S-O-S®, for which the MIC values were found to be 38 mg/mL or 55 mg/mL, depending on the bacterial strain used in MIC determination.[19] It seems worth emphasizing that apart from antibacterial properties, the fruit extracts studied also showed some anti-inflammatory properties and they appeared to be efficient free radical scavengers and antioxidant agents.

Conclusions

A significant finding in the current work is that it describes the antioxidant, antiradical, anti-inflammatory, and antimicrobial properties of polyphenol-rich extracts. The analyzed plants belonging to the *Rosaceae* family may be considered as valuable agents protecting in vitro the model lipid membrane against peroxidation and can reduce the risk of urinary tract infections. Japanese quince and dog rose extracts in particular, showing very promising biological properties, seem to be able to join the list of substances that can be used as dietary supplements aimed at preventing, for example, urinary tract infections, or as supportive of drug treatment in many diseases.

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Age and gender differences in clinical outcomes of patients with heavy-calcified coronary artery lesions treated percutaneously with rotational atherectomy

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Abstract

Background. Rotational atherectomy (RA) used in elderly patients treated with percutaneous coronary interventions (PCI) could enable revascularization or the omission of cardiac surgery. Knowledge about factors affecting the prognosis may improve the results of treatment.

Objectives. We aimed to assess the relationship of gender and age with long-term clinical outcomes expressed as major adverse cardiac and cerebrovascular events (MACCEs).

Material and methods. The study included 97 consecutive patients treated with PCI and RA at the mean age of 71. The study group contained 73.2% men and 26.8% women, 36.1% of patients older than 75 and 63.9% younger than 75. The mean time of follow-up was 695.3 ±560.9 days. The rate of MACCEs (deaths, myocardial infarctions (MIs), reinterventions, coronary artery by-pass surgeries, or cerebral strokes (CSs)/transient ischemic attacks (TIAs)) in the overall group of patients was calculated at 33.7%.

Results. The comparison of Kaplan–Meier survival curves did not depict significant differences in the frequency of MACCEs for age ($p = 0.36$) and gender ($p = 0.07$). We noticed that the death rate was higher in females than in males and in patients older than 75 compared to those younger, and was statistically significant for age ($p = 0.04$). The rate of periprocedural complications was significantly higher among women than among men ($p = 0.005$) and in patients older than 75 compared to the younger ones ($p = 0.003$).

Conclusions. Age and gender are not significantly associated with an increased rate of MACCEs during follow-up in elderly patients treated with PCI and RA.

Key words: age, predictors, gender, clinical outcomes, rotational atherectomy

Introduction

In the era of population aging and the increase in the percentage of elderly patients, differences in the therapeutic approach among this group of patients are of special importance. A number of published studies have shown that age is associated with increased mortality and worse prognosis in the follow-up period among hospitalized patients.[1] Poorer prognosis in the elderly is associated with several factors reflecting the functional changes in their organisms. Some researchers even attempted to identify the most important independent risk factors and created a prognostic index for this group of patients to estimate the probability of mortality, and thus, the possibility of interfering with these factors to prolong life in this group of patients.[2] One of the independent risk factors of increased mortality in patients after hospitalization is ischemic heart disease. Previously published studies have shown differences in response to the established treatment regimens in the elderly group of patients, and that appropriate modification of the diagnostic and therapeutic processes in this group of patients may contribute to a significant improvement in treatment outcomes compared to younger patients.[3,4] Increased calcification in coronary arteries is associated with a higher risk of ischemic heart disease and cardiovascular events as well as mortality in elderly patients.[4] Rotational atherectomy (RA) as a device to facilitate percutaneous coronary interventions (PCI) is dedicated to patients with heavy calcified stenoses in the coronary arteries.[5] The RA is applicable to elderly patients, although the number of publications comparing the results of its use depending on age is limited. Several studies have shown that age is an independent risk factor for major adverse coronary events in this group of patients.[6] In comparative studies, the relationship of gender and long-term treatment results in the case of orbital atherectomy in the group of patients with heavy calcified lesions in coronary arteries has not been demonstrated, while the number of publications on RA is very limited.[7]

Therefore, in the current study, we aimed to assess the relationship of gender and age with long-term clinical outcomes in patients treated with PCI and RA expressed as major adverse cardiac and cerebrovascular events (MACCE).

Material and methods

Our study was a retrospective and partially prospective registry of 97 consecutive patients undergoing PCI with RA from 2002 to 2017 in the primary reference center. The majority of patients were included during the last 10 years. All patients were screened for risk factors, concomitant diseases, past PCI history, and medications. Procedural details, and in-hospital and long-term complications were collected. The maximum follow-up period was 2,315 days and the study endpoints were the rates of MACCE defined as the following: death (overall mortality), the requirement of target lesion revascularization expressed as endovascular reintervention or coronary artery by-pass grafting (CABG) operation, myocardial infarction (MI), and cerebral stroke (CS)/transient ischemic attack (TIA). All patients gave informed consent for the procedure. The study was approved by the institutional ethical board and conformed to the ethical principles for medical research involving human subjects of the 1975 Declaration of Helsinki.

Procedure

Patients were qualified for the procedure according to the current guidelines.[5] A Rotablator (Boston Scientific, Marlborough, USA) was used to perform the procedure. We evaluated the preprocedural risk with Euroscore II and Syntax score online calculators (http://euroscore.org/calc.html and http://www.syntaxscore.com/calculator/start.htm respectively). The risk was defined as: low (≤2%), intermediate (3–5%) and high (≥5%) for the EUROSCORE II and low (0–22 points), intermediate (23–32 points) and high (≥33 points) for the SYNTAX score.

Procedure-related complications

The rate of procedure-related complications consisted of the intraprocedural complications and periprocedural hospitalization, which occurred after the procedure until discharge from the hospital. The procedure-related MI was defined according to the 3rd universal definition of MI.[8] Coronary artery perforation was defined according to the most common and recognized definition proposed by Ellis at al.[9] The procedure-related hematoma was included in the analysis when it demanded surgical intervention or/and blood products transfusion. Cardiogenic shock was defined according to the current European Society Guidelines.[10] We included all the deaths that occurred during the procedure or after the procedure until discharge from the hospital into the current analysis as overall mortality. The allergic reaction was found to be significant when it demanded prolonged hospitalization and parenteral or oral therapy with antiallergic specifics. Contrast-induced nephropathy was recognized according to the definition published by Mehran et al.[11] Coronary artery dissection was defined according to the most common classification.[12] Other technical complications associated with the RA procedure itself were reported in accordance with current recommendations regarding the use of RA in Europe.[13] For periprocedural arrhythmias, we included persistent ventricular and supraventricular tachyarrhythmias, and atrioventricular conductive disorders requiring additional pharmacological or interventional treatment (pacing or cardioversion). Slow-flow or no-reflow was defined as TIMI (thrombolysis in myocardial infarction) flow grade 0 or 1.[14]

Statistical analysis

Normal distribution was assessed with the Shapiro–Wilk test. The χ^2 test was used to compare the categorical variables. For comparisons of continuous data, the Mann–Whitney U test or the Student's t-test were performed where applicable. The log-rank test was performed for comparisons of Kaplan–Meier survival curves in selected subgroups. Univariate analysis of selected potential predictors of clinical outcomes defined as MACEEs was also performed. A probability of $p < 0.05$ was considered statistically significant. The statistical analyses were conducted with STATISTICA v. 10.0 software (StatSoft, Inc., Tulsa, USA) and SPSS Statistics v. 24 (IBM Corp., Armonk, USA).

Results

The overall group of patients included 97 subjects in which successful rotablation was performed. Among them, there were 71 males (73.2%) and 26 females (26.8%), 62 individuals older than 75 (63.9%) and 35 patients younger than 75 (36.1%). The distribution of patients within particular age groups depending on gender is presented in Fig. 1.

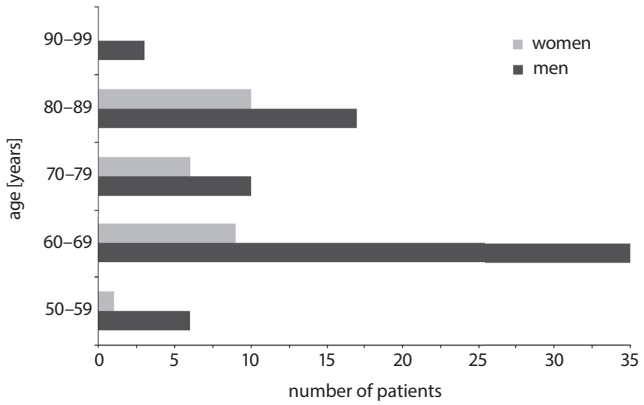

Fig. 1. Patient distribution in selected age intervals according to gender

General characteristics

The mean age of the males was 70.8 ±10 years and females 73.8 ±9.2 years. Males were significantly more often smokers (69.6% vs 10%, p = 0.03) and their mean ejection fraction of the left ventricle (LVEF) was smaller (43.9% vs 51.4%, p = 0.036), while the only 2 cases qualified for the RA in salvage mode were females (p = 0.01).

Patients older than 75 had significantly smaller mean body mass index (BMI) values (26.7 kg/m^2 vs 29.2 kg/m^2; p = 0.04), and presented at admission significantly more often with unstable angina (UA; 28.3% vs 53.1%, p = 0.019) or non-significantly less often with stable angina and non-ST segment elevation MI (NSTEMI). They also were qualified significantly more often for the procedure in emergency mode (26.5% vs 6.9%, p = 0.002) and non-significantly less often in elective and urgent mode. Moreover, the older group of patients characterized with greater mean serum creatinine level (p = 0.005) and smaller glomerular filtration rate (GFR) level (p < 0.001). All clinical data are presented in Table 1.

The mean EUROSCORE II value was higher in females compared to males; however, it was without statistical significance (3.3% ±3.3% vs 9.4% ±18.4%, p = 0.4). The higher mean EUROSCORE II value was attributed to the fact that among females there were 2 extremely high-risk patients treated in salvage mode, which increased the mean value. A similar relation was found when the overall group was divided according to age, where the 2 patients were placed in the group of patients older than 75. The difference in the mean EUROSCORE II value between patients younger and older than 75 was significant (9.2% ±15.2% vs 2.3% ±2.8%, p < 0.001). In the case of gender division, males were more often at low risk (44.6%) compared to moderate (35.4%) and high-risk patients (0.2%), and in comparison to women, each risk group did not differ significantly. Among women, the distribution in the low (34.8%), moderate (34.8%) and high-risk (30.4%) cases was more even.

In patients younger than 75, there were more patients in the low-risk group (58.2%) in comparison to moderate (50.9%) and high-risk (9.1%) groups. Not surprisingly, opposite distribution was shown in patients older than 75, among whom most patients were in the high-risk group (45.4%), while in the moderate (39.4%) and low-risk (15.1%) groups, their number was smaller. The difference between younger and older patients was significant for the low-risk group (p = 0.001) and high-risk group (p < 0.001).

Pharmacotherapy

There were no statistically significant differences in pharmacological treatment between males and females. Patients older than 75 were treated significantly more often with clopidogrel as a second antiplatelet drug (83% vs 100%, p = 0.01). This was mainly due to more frequent use of ticagrelor (8.3% vs 0%, p = 0.08) and prasugrel (6.7% vs 0%, p = 0.12) in younger patients. The frequency of other medications did not differ significantly between patients younger and older than 75. This is presented in Table 2.

Lesion characteristics and procedural indices

The mean SYNTAX Score I value did not differ significantly between males and females, and was greater in females (24.1 ±13 points vs 26.3 ±13.0 points, p = 0.45). Patients older than 75 were presented with greater mean SYNTAX Score I value compared to younger patients; however, the difference did not reach statistical significance (28.1 ±13.4 points vs 22.1 ±13 points, p = 0.06). When comparing SYNTAX Score I groups, there were

Table 1. General characteristics

Variable	Gender		p-value	Age [years]		p-value
	male	female		<75	≥75	
Age [years]	70.8 ±10.0	73.8 ±9.2	0.13	65.4 ±5.8	82.6 ±4.0	<0.000
BMI [kg/m^2]	27.9 ±6.3	28.9 ±4.6	0.50	29.2 ±7.0	26.7 ±3.1	0.04
Gender (males)	–	–		49 (79)	22 (62.8)	0.08
Diabetes	34 (49.3)	15 (60)	0.36	34 (56.7)	15 (44.1)	0.31
Hypertension	66 (95.6)	25 (100)	0.29	57 (95)	34 (100)	0.68
Hyperlipidemia	65 (94.2)	25 (100)	0.22	57 (95)	33 (97.1)	0.86
Obesity	18 (27.3)	6 (26.1)	0.91	18 (32.1)	6 (18.2)	0.27
Prior PCI	47 (73.4)	13 (61.9)	0.31	41 (75.9)	19 (61.3)	0.26
Prior MI	46 (66.7)	15 (65.2)	0.92	32 (59.2)	25 (73.5)	0.28
Prior CABG	10 (14.7)	4 (16)	0.87	11 (18.6)	3 (8.8)	0.43
PAD	19 (27.9)	3 (12)	0.11	16 (27.1)	6 (17.6)	0.44
COPD	9 (13)	5 (20)	0.40	7 (16.7)	7 (20.6)	0.24
CS/TIA	10 (14.5)	4 (16)	0.85	5 (8.3)	9 (26.5)	0.14
Heart failure	23 (33.3)	8 (32)	0.90	19 (31.7)	12 (35.3)	0.72
Smoking	48 (69.6)	10 (40)	0.03	38 (63.3)	20 (58.8)	0.71
Kidney failure	13 (18.8)	5 (20)	0.93	8 (13.3)	10 (29.4)	0.06
Clinical picture						
stable angina	36 (54.5)	9 (36)	0.11	32 (53.3)	14 (43.7)	0.38
UA	17 (25.7)	8 (32)	0.55	17 (28.3)	17 (53.1)	0.019
NSTEMI	13 (19.7)	7 (28)	0.39	9 (15.0)	1 (3.1)	0.08
STEMI	3 (4.5)	1 (4)	0.90	2 (1.7)	0 (0)	0.29
Mode						
elective	39 (56.5)	10 (40)	0.15	35 (60.3)	14 (41.2)	0.10
urgent	21 (30.4)	9 (36)	0.60	21 (36.2)	9 (26.5)	0.39
emergency	9 (13)	4 (16)	0.71	4 (6.9)	9 (26.5)	0.002
salvage	0 (0)	2 (8)	0.01	0 (0)	2 (5.9)	0.06
LVEF [%]	43.9 ±12.7	51.4 ±10.8	0.036	47.8 ±11.5	42.3 ±13.9	0.13
GFR [mL/min]	80.0 ±33.2	64.2 ±29.4	0.08	92.4 ±28.4	48.9 ±17.6	<0.000
Creatinine [µmol/L]	92.5 ±33.3	85.6 ±25.7	0.21	83.6 ±20.5	103.2 ±42.0	0.005

BMI – body mass index; CABG – coronary artery by-pass grafting; COPD – chronic obstructive pulmonary disease; CS – cerebral stroke; GFR – glomerular filtration rate; LVEF – left ventricle ejection fraction; MI – myocardial infarction; NSTEMI – non ST-segment elevation MI; PAD – peripheral artery disease; PCI – percutaneous coronary intervention; STEMI – ST-segment elevation MI; TIA – transient ischemic attacks; UA – unstable angina.

Table 2. Pharmacotherapy at discharge

Variable	Gender		p-value	Age [years]		p-value
	male	female		<75	≥75	
ASA	71 (100)	26 (100)	–	62 (100)	35 (100)	–
Antiplatelets						
clopidogrel	59 (88)	25 (96.2)	0.18	50 (83)	34 (97.1)	0.01
ticagrelor	4 (6)	1 (3.8)	0.69	5 (8.3)	0 (0)	0.08
prasugrel	4 (6)	0 (0)	0.20	4 (6.7)	0 (0)	0.12
ticlopidine	1 (1.5)	0 (0)	0.53	1 (1.7)	0 (0)	0.44
Oral antidiabetic	18 (26.1)	6 (25)	0.93	21 (35)	3 (9.1)	0.03
Insulin therapy	18 (26.1)	8 (32)	0.66	18 (30)	8 (23.5)	0.60
ACEis/ARB	33 (47.8)	10 (41.7)	0.65	27 (45)	16 (48.5)	0.78
Diuretic	34 (55.7)	10 (50)	0.95	23 (45.1)	21 (70)	0.28
β-blocker	64 (92.7)	24 (100)	0.59	56 (93.3)	32 (97)	0.77
Statin/fibrate	68 (98.5)	23 (95.8)	0.91	57 (96.6)	34 (100)	0.89
Anticoagulant	14 (20.3)	6 (25)	0.72	8 (13.3)	12 (36.4)	0.06
Nitrate	4 (5.8)	2 (8.3)	0.85	2 (3.3)	4 (12.1)	0.48
Ca-blocker	20 (29)	9 (37.5)	0.35	19 (31.7)	10 (30.3)	0.91

ASA – acetyl-salicylic acid; ACEi – angiotensin-converting enzyme inhibitors; ARB – aldosterone receptor blockers.

no significant differences between males and females in the low (45.6% vs 35.7%, p = 0.51), intermediate (28.2% vs 28.6%, p = 0.98) and advanced lesions (26.1% vs 35.7%, p = 0.48). While considering patients in terms of age, there were significantly more patients with less advanced atherosclerotic lesions in the coronary arteries among patients younger than 75 compared to older patients (54.3% vs 28%, p = 0.04), whereas there was not a significantly greater percentage of patients older than 75 in the intermediate (36% vs 22.8%, p = 0.26) and high (36% vs 22.8%, p = 0.26) SYNTAX Score I group compared to younger patients.

Both in the case of the division into women and men and into patients over and under the age of 75, atherosclerotic lesions in type C according to American Heart Association (AHA) classification were more frequent in comparison to type 2B lesions. Type C lesions occurred not significantly more often in males than in females (78.7% vs 71.4%, p = 0.56), and in patients older than 75 (84% vs 73.5%, p = 0.33), while type 2B lesions occurred more often in females (28.6% vs 21.3%, p = 0.56) and patients younger than 75 (26.5% vs 16%, p = 0.33).

Females were treated significantly more often from right radial access compared to males (50% vs 25.9%, p = 0.04), while patients older than 75 were treated significantly less often with single stent implantation compared to younger patients (80% vs 60%, p = 0.03), and insignificantly more often with 2 or 3 stents. Lesion characteristics and procedural indices are presented in Table 3.

Significantly more procedure-related complications occurred in females compared to males (46.1% vs 18.3%, p = 0.005). Also, older patients (>75 years) demonstrated a significantly higher frequency of procedure-related complications (42.8% vs 16.1%, p = 0.003). More detailed characteristics are presented in Table 4.

Table 3. Lesion characteristics and procedural indices

Variable	Gender		p-value	Age [years]		p-value
	male	female		<75	≥75	
Direct indication was inability to:						
cross with b.c.	27 (40.3)	8 (34.8)	0.63	21 (36.2)	14 (43.7)	0.48
dilate with b.c.	39 (58.2)	12 (52.2)	0.61	34 (58.6)	17 (53.1)	0.61
stent delivery	1 (1.4)	3 (13)	0.02	3 (5.2)	1 (3.1)	0.65
Mean burr diameter [mm]	1.49 ±0.20	1.43 ±0.30	0.26	1.47 ±0.20	1.47 ±0.20	0.57
Maximum burr diameter [mm]	1.5 ±0.20	1.4 ±0.20	0.27	1.48 ±0.20	1.48 ±0.30	0.69
Arteries						
1	56 (78.9)	16 (64)	0.13	49 (80.3)	23 (65.7)	0.11
2	12 (16.9)	7 (28)	0.23	10 (16.4)	9 (25.7)	0.26
3	3 (4.2)	2 (8)	0.46	2 (3.3)	3 (8.6)	0.46
Stents						
1	36 (54.5)	15 (60)	0.63	49 (80)	21 (60)	0.03
2	21 (31.8)	7 (28)	0.72	10 (16.4)	10 (28.6)	0.15
3	8 (12.1)	2 (8)	0.57	2 (3.3)	4 (11.4)	0.11
4	1 (1.5)	1 (3.6)	0.48	0 (0)	0 (0)	–
Stent length [mm]	26.2 ±10.2	24.0 ±8.5	0.51	26.6 ±11.0	24.1 ±7.6	0.49
Stent diameter [mm]	3.04 ±0.70	3.06 ±0.80	0.90	3.05 ±0.70	3.0 ±0.70	0.62
Balloon diameter before PCI [mm]	2.9 ±0.5	2.6 ±0.6	0.06	2.9 ±0.6	2.7 ±0.5	0.27
Post-dilatation	58 (87.9)	19 (76)	0.38	51 (89.5)	26 (76.5)	0.30
Balloon diameter after PCI [mm]	3.6 ±0.7	3.7 ±1.0	0.93	3.6 ±0.7	3.6 ±0.9	0.50
IVUS after PCI	18 (25.3)	3 (11.5)	0.29	15 (24.2)	6 (17.1)	0.56
Radiation dose [Gy]	1.97 ±1.20	1.9 ±1.2	0.77	2.04 ±1.40	1.8 ±0.8	0.89
Contrast volume [mL]	271 ±106	303 ±140	0.53	285 ±140	272.0 ±69.6	0.69
Type of PCI						
POBA/DEB	3 (4.4)	0 (0)	0.28	3 (5.2)	0 (0)	0.17
DES	64 (94.1)	24 (96)	0.72	55 (94)	33 (94.3)	0.91
BMS	1 (1.5)	1 (4)	0.45	0 (0)	2 (5.7)	0.06
Vascular access						
RRA	15 (25.9)	10 (50)	0.04	16 (34.8)	9 (28.1)	0.53
LRA	2 (3.4)	0 (0)	0.40	1 (2.2)	1 (3.1)	0.79
RFA	36 (62.1)	9 (45)	0.18	24 (52.2)	21 (65.6)	0.23
LFA	5 (8.6)	1 (5)	0.60	5 (10.9)	1 (3.1)	0.20

b.c. – balloon catheter; BMS – bare-metal stent; Cx – circumflex branch; DEB – drug-eluting balloon; DES – drug-eluting stent; Dg – diagonal branch; IVUS – intravascular ultrasound; LAD – left anterior descending branch; LFA – left femoral artery; LMCA – left main coronary artery; LRA – left radial artery; Mg – marginal branch; PCI – percutaneous coronary intervention; POBA – plain-old balloon angioplasty; RCA – right coronary artery; RFA – right femoral artery; RRA – right radial artery.

Table 4. Procedure-related complications

Variable	Gender		p-value	Age [years]		p-value
	male	female		<75	≥75	
Overall rate	13 (18.3)	12 (46.1)	0.005	10 (16.1)	15 (42.8)	0.003
AD	3 (4.2)	2 (7.7)	0.49	3 (4.8)	2 (5.7)	0.85
CS	1 (1.4)	3 (11.5)	0.02	2 (3.2)	2 (5.7)	0.55
MI	2 (2.8)	1 (3.8)	0.79	2 (3.2)	1 (2.8)	0.91
Death	0 (0)	1 (3.8)	0.09	0 (0)	1 (2.8)	0.18
Bleeding	1 (1.4)	2 (7.7)	0.11	1 (1.6)	2 (5.7)	0.27
Hematoma	1 (1.4)	2 (7.7)	0.11	0 (0)	3 (8.6)	0.02
Rotawire disruption	0 (0)	1 (3.8)	0.09	0 (0)	1 (2.8)	0.18
Arrhythmias	2 (2.8)	0 (0)	0.38	1 (1.6)	1 (2.8)	0.67
Allergic reaction	1 (1.4)	0 (0)	0.54	1 (1.6)	0 (0)	0.45
Slow flow	1 (1.4)	0 (0)	0.54	0 (0)	1 (2.8)	0.18
CIN	1 (1.4)	0 (0)	0.54	0 (0)	1 (2.8)	0.18

AD – arterial dissection; CIN – contrast-induced nephropathy; CS – cerebral stroke; MI – myocardial infarction.

Clinical outcomes and follow-up

Follow-up was completed among the overall study group in 78 (80.4%) patients included into the study. During the mean time to the MACCE or follow-up without MACCE (695 ±560 days), MACCEs occurred in 26 patients (33.7%). Among the MACCEs, there were 14 deaths (53.8%), 4 reinterventions (15.4%), 5 MIs (19.2%), 2 CS/TIA cases (7.7%), and 1 coronary artery by-pass grafting (CABG) operation (3.8%).

The frequency of MACCEs was greater among females compared to males, however, without statistical significance (45.4% vs 28.6%, p = 0.32). This was mainly due to greater rate of reinterventions (9.1% vs 3.6%, p = 0.7) and deaths (27.3% vs 14.3%, p = 0.37). Also, the MACCEs rate was greater in patients older than 75 compared to those younger than 75 (42.8% vs 28.6%, p = 0.38), and this was mainly the consequence of greater rate of deaths among older patients (35.7% vs 8%, p = 0.04). This is presented in Table 5.

The Kaplan–Meier survival curve comparison between males and females confirmed that, despite the fact that the frequency of MACCEs was higher in women, there was no significant difference between those 2 groups (p = 0.07). This is presented in Fig. 2.

Similarly, the greater rate of MACCEs in patients older than 75 compared to those younger did not meet statistical significance in comparison of the Kaplan–Meier survival curves (p = 0.36). This is presented in Fig. 3.

Predictors of clinical outcomes

Univariate analysis of several selected factors revealed that among them, statistically significant relationships with MACCEs were achieved for the maximal burr diameter (p = 0.03), the occurrence of procedure-related complications (p = 0.015), the mean EUROSCORE II value (p = 0.02), and borderline relationship with obesity (p = 0.052). Considering the fact that age and gender were

Table 5. Study endpoints and duration of follow-up in selected groups of patients

Variable	Gender		p-value	Age [years]		p-value
	male	female		<75	≥75	
Completed follow-up	56 (78.9)	22 (84.6)	0.90	50 (80.6)	28 (80)	0.88
Mean time of follow-up [days]	727 ±536	614 ±625	0.18	677 ±541	726 ±603	0.80
MACCE	16 (28.6)	10 (45.4)	0.32	14 (28.6)	12 (42.8)	0.38
Mean time to MACCE [days]	638 ±573	324 ±435	0.09	574 ±634	451 ±415	0.95
Re-PCI	2 (3.6)	2 (9.1)	0.70	4 (8)	0 (0)	0.55
Death	8 (14.3)	6 (27.3)	0.37	4 (8)	10 (35.7)	0.04
CS/TIA	1 (1.8)	1 (4.5)	0.85	1 (2)	1 (3.6)	0.90
MI	4 (7.1)	1 (4.5)	0.34	4 (8)	1 (3.6)	0.19
CABG	1 (1.8)	0 (0)	0.90	1 (2)	0 (0)	0.88

CABG – coronary artery by-pass grafting; CS – cerebral stroke; MACCE – major adverse cardiac and cerebrovascular events; MI – myocardial infarction; PCI – percutaneous coronary intervention; TIA – transient ischemic attack.

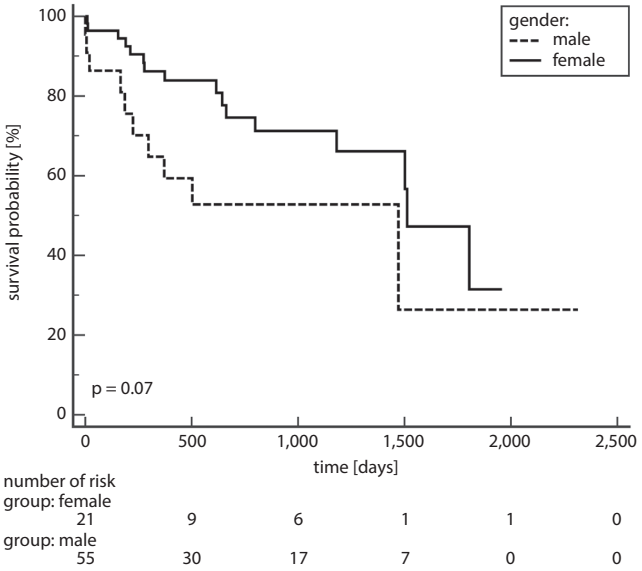

Fig. 2. The comparison of Kaplan–Meier survival curves according to gender

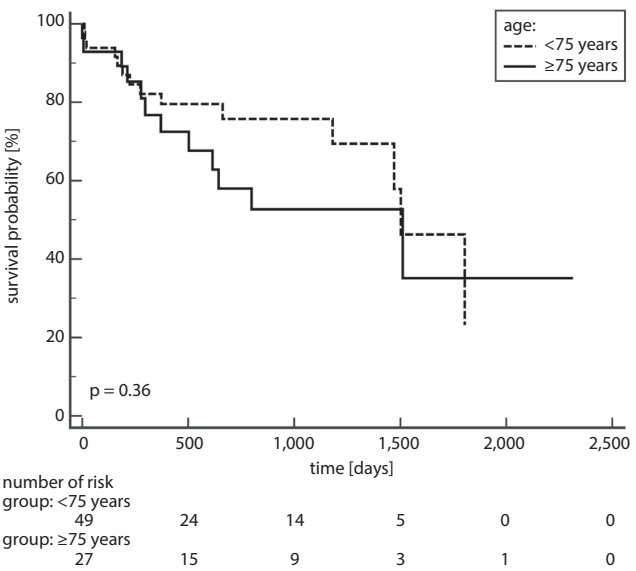

Fig. 3. The comparison of Kaplan–Meier survival curves according to the age of 75

not among the factors associated with the occurrence of MACCE in the follow-up period, a multivariate analysis was not performed.

Discussion

The crucial finding of the presented analysis is that gender and age greater than 75 were not found to be significantly related to poorer clinical outcomes expressed as MACCE rate during the long-term follow-up period compared with survival curves. However, it should be noted after the analysis of individual components of MACCEs, for both women and patients in the age group above 75, the incidence of deaths was higher, and in the case of age-related differences, mortality was statistically significantly greater in the group of older patients. The univariate analysis did not confirm the relationship of age and gender with MACCEs during the follow-up period. Instead, it revealed that the maximal burr diameter, EUROSCORE II value and the presence of procedure-related complications are associated with long-term clinical outcomes after RA. The relationship between the EUROSCORE II value and the length of the implanted stent was demonstrated in a previously published paper.[15] The level of perforations in the presented study was higher (3.1%) when compared to the published results based on large registries (0.3–1.1%).[16,17] However, coronary perforations observed in our analysis were not complicated by serious and life-threatening incidents, including cardiac tamponade and surgical treatment. They were treated successfully with an endovascular approach. Moreover, usually registries, including the Polish National Registry, underestimate the rate of procedure-related complications.[18,19] The frequency of the overall procedure-related complication rates was significantly greater in females and in the group of patients older than 75. In the case of age, this was mainly due to the greater number of arterial dissections, cardiogenic shocks and MIs, while in the case of the female gender, arterial dissections, cardiogenic shocks, arterial perforations, and hematomas demanding blood transfusion or surgical treatment were more frequent.

Some published studies estimating the relationship between age and long-term clinical outcomes in patients with heavy calcified coronary artery lesions demonstrated that age is an independent risk factor of poorer clinical outcomes expressed as MACCE.[6] However, most of them did not confirm a significant relationship between age and long-term clinical outcomes, and among the factors that have independent influence on the prognosis, they confirmed LVEF, diabetes, cardiogenic shock, acute coronary syndrome, kidney failure, SYNTAX score, neutrophil/lymphocyte ratio, burr to artery ratio, or prior PCIs.[20–23] Most of these studies involved several hundreds of patients. Considering the various components of MACCE, Abdel-Wahab et al. demonstrated that age was an independent predictor of target lesion revascularization.[20] Our study included only about 100 participants, which makes it one of the smaller studies among those discussed. As a consequence, the results are very susceptible to bias. One of the factors that certainly influenced the bias was the severe state of some patients qualified for RA at a young age for the present cohort of patients, which were the causes of MACCE occurrence during the hospital stay or shortly after discharge. Operators are less likely to perform heroic rotablation treatments in older patients with multiple risk factors, which undoubtedly influenced the artificial masking of mortality in the early periprocedural stage in patients over 75.

Moreover, we did not break down MACCE into individual components in order to assess the impact of age and gender in survival curve analysis on long-term treatment results due to an insufficient number of endpoints in individual groups of patients and the resulting high probability of error and bias. However, we performed such a comparison of study endpoints for both groups and showed almost twice as high mortality in the follow-up period in women and patients over the age of 75, although this difference was statistically significant only for older patients. Nonetheless, in the case of women, this relationship can be explained by the higher mean value of the initial EUROSCORE II, which was mainly associated with a greater number of salvage mode procedures, greater mean age and more procedure-related complications. On the other hand, it should also be noted that women initially had a statistically significantly higher mean LVEF and they smoked cigarettes less frequently, which in some way influenced mortality balance and made it statistically non-significant. In the case of older age, the mortality rate was statistically significantly higher in the group of patients older than 75, which was in line with other factors closely related to increased mortality as serum creatinine, GFR, mean SYNTAX score value, or mean EUROSCORE II value. In addition, the age of the patients and the aging of the body were also of great importance, as exemplified by the occurrence of deaths in patients above 90 years of age after a period of just over 200 days of the follow-up period. There are no publications attainable in the available literature that closely compare the long-term clinical outcomes of patients with severely calcified lesions of coronary arteries using RA. On the other hand, studies estimating the influence of individual factors on the results of treatment did not show that gender was related to treatment outcomes presented as MACCE prevalence. One of the few available studies on the treatment of similar coronary artery lesions, but undertaken with the use of another device, regards orbital atherectomy.[7] This study was conducted on a relatively large group of patients including 458 participants. The incidence of MACCE did not differ significantly between the 2 groups and was 0.7% and 2.7%, respectively, for women and men ($p = 0.14$). Admittedly, the incidence of MACCE in this study was higher in men than women. One of the factors that could have an impact on the incidence of MACCE in this study was higher mean length and diameter of stents in men compared to women, which was shown to be related to the incidence of MACCE in the follow-up period in other studies, including those published by our center.[15] On the other hand, in this study, the men were statistically significantly younger and less likely to have hypertension and hypercholesterolemia. Men were also insignificantly younger in our study, which certainly influenced the results.

Considering the relationship of procedure-related complications with gender and age, it turned out that the incidence of complications in both women and older individuals is greater. Already in a previously published publication by our center, we demonstrated on a large group of patients that the incidence of arterial perforations was related to age, and that age is an independent predictor of increased risk of their occurrence,[18] which is in contrast, for example, to acute coronary syndromes that have not been shown to be significantly associated with the incidence of procedure-related complications.[24] The increased rate of procedure-related complications is mainly caused by calcifications in the coronary arteries, which predispose to typical complications of RA such as dissections or perforations.[16,25] Previously published studies on the association of gender with periprocedural complications treated with PCI indicate a greater susceptibility of women to procedure-related complications.[26] This trend also seems to be sustained for PCI with RA.

Conclusions

The negative relationship of age and gender with clinical outcomes expressed as increased rate of MACCEs in elderly patients treated with PCI and RA does not have as much impact as in the case of patients in other age groups, which should encourage operators to reduce their concerns in the treatment of patients over 75 years of age with rotablation.

Study limitations

The results of the presented work should be received with great caution, because the influence of age on poorer results in the follow-up period seems to be evident in all groups of patients treated with percutaneous coronary angioplasty, although the purpose of this article is to show that well-prepared and stable patients among the elderly may achieve comparable benefits from the use of rotablation compared to younger patients. In the current work, apart from a very small group of patients as for this type of treatment and a relatively large number of patients lost in the follow-up period, there are a number of factors that artificially mask the actual impact of age and sex on long-term treatment results. One of the main factors is undoubtedly the greater willingness of the use of rotablation in the salvage mode, especially in younger and promising patients, compared to the older and less promising individuals burdened with many concomitant diseases, including cancer, heart failure and others, which artificially increased the number of deaths in the early periprocedural stage in patients under 75 years of age.

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Analysis of the clinical response and changes in the expression of TNF-α and its TNFR1 and TNFR2 receptors in patients with psoriasis vulgaris treated with ustekinumab

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Abstract

Background. Ustekinumab is a monoclonal antibody that shows the ability to bind to subunit p40, common for interleukin 12 (IL-12) and IL-23, which prevents the activation of the JAK STAT signaling pathway.

Objectives. The objective of the study was to evaluate the efficacy of therapy that uses anti-IL-12/23 medicine in patients with psoriasis vulgaris, based on the disease clinical progression indices (Psoriasis Area and Severity Index (PASI), Dermatology Life Quality Index (DLQI) and Body Surface Area (BSA)) and to determine the possibilities of using changes in the expression profiles of tumor necrosis factor α (TNF-α), tumor necrosis factor receptor (TNFR1) and TNFR2 as molecular markers showing the response to ustekinumab therapy.

Material and methods. The group under study was composed of 14 patients (10 men and 4 women, aged 49.3 ±10.2 years) with diagnosed psoriasis vulgaris, treated with ustekinumab. The group was divided into subgroups because of the selected 3 stages of therapy. The control group consisted of 20 healthy volunteers (11 men and 9 women, aged 46 ±10 years). The 120-week long observation involved a clinical assessment of the patients (PASI, BSA and DLQI), based on the following scheme: 0–4–12 weeks of the observation. The analysis of molecular changes in the TNF-α, TNFR1 and TNFR2 expression profiles was performed with the quantitative reverse-transcription polymerase chain reaction (RT-qPCR) method, using the patients' full blood. The statistical analysis was performed with STATISTICA v. 12.0 PL (StatSoft, Inc., Tulsa, USA) with the level of statistical significance $p < 0.05$.

Results. Gradually reduced PASI, BSA and DLQI values were observed during anti-IL-12/23 therapy. An increased level of the TNF-α transcription activity was observed in the analyzed group when compared to the control. Correlations between the clinical and molecular parameters were also indicated.

Conclusions. Ustekinumab constitutes an efficient and safe form of pharmacotherapy in psoriasis vulgaris. We did not observe any reduced efficacy of the treatment when reclassifying patients for the therapy. Tumor necrosis factor α, TNFR1 and TNFR2 may serve as supplementary markers of molecular response to the medicine.

Key words: psoriasis, TNF-α, molecular marker, anti-IL-12/23 therapy, JAK STAT signaling pathway

Introduction

Psoriasis is a multi-factor pro-inflammatory disease, the most characteristic phenomenon of which is parakeratosis.[1–3] Moreover, the disease involves the increased risk of concurrent metabolic syndrome, arterial hypertension, cardiovascular diseases, and ischemic brain stroke.[2,4–6] Development of molecular biology made it possible to observe and confirm the changes occurring in the expression of cytokines, e.g., transforming growth factor β (TGF-β), tumor necrosis factor α (TNF-α) and interleukins (IL-12, IL-23 and IL-17) in the said disease.[2,7]

Biological anti-cytokine therapy is intended for those patients for whom conventional therapies proved to be insufficiently effective or in cases where contraindications for their application occurred. Moreover, in this group of patients, psoriasis has got a severe course and a high degree of lesion advancement (Psoriasis Area and Severity Index (PASI) >10 or Body Surface Area (BSA) >10).[7–9]

Two big groups of anti-cytokine medicines are distinguished: TNF-α inhibitors (adalimumab, etanercept) and IL-12/23 inhibitor (ustekinumab),[10,11] which owe their high efficacy to their unique activity: their focus on molecular objectives.[10–12]

When binding to the mentioned subunit p40, ustekinumab prevents IL-12/23 from interacting with the receptor and activation of the JAK STAT signaling pathway and is also a monoclonal antibody oriented against the p40 subunit, common for IL-12 and IL-23.[1,8]

Therapy based on this medicine is comparably efficient in anti-TNF therapy and enables us to achieve remission of disease symptoms.[1,8,13]

Interaction between IL-12 and IL-23 and receptors on the cell surface activates the JAK STAT signaling pathway,[14,15] the final products being the following: TNF-α and interferon gamma (IFNγ),[16] which enhance IL-12/23 secretion,[17] thereby causing intensified inflammation.[18–20]

Tumor necrosis factor α is mainly generated by the following: macrophages, monocytes, T and B lymphocytes, and its interactions with receptors *TNFR1* and *TNFR2* trigger activation of apoptosis or the NFκB pathway.[21]

To clinically assess the changes in patient, advancement of disease and efficacy of therapy in psoriasis vulgaris, the following scales are used: PASI, Dermatology Life Quality Index (DLQI) and BSA.[22]

Objectives

The objective of the study was to evaluate the efficacy of anti-IL-12/23 therapy in patients with psoriasis vulgaris, based on the clinical parameters (PASI, DLQI and BSA), and to analyze the transcription activity profiles of *TNF-α*, *TNFR1* and *TNFR2*. The possibility of using the changes in the expression of the analyzed genes as supplementary molecular markers of therapy efficacy was also determined.

Material and methods

Material used for the studies involved blood taken from 14 patients with psoriasis, classified for ustekinumab therapy. The blood was sampled before the administration of an ustekinumab dose and 2 h after its administration (one stage of the therapy lasted 40 weeks). The analyzed group was divided into 3 subgroups, as the patients had to be reclassified to a given pharmacotherapy program and a new stage of therapy had to be initiated: subgroup I included 14 patients (10 men and 4 women, aged 49.3 ±10.2 years), subgroup II included 9 patients (7 men and 2 women, aged 50.1 ±7.64 years) and subgroup III included 5 patients (3 men and 2 women, aged 52.8 ±7.98 years). The interval between the subsequent inclusions to therapy was 4–6 months. The need to reclassify the patients to anti-cytokine therapy resulted from the criteria of IL-12/23 therapy inclusion and from whether any of the patients show remission. This is due to the fact that, in accordance with the Polish guidelines for anti-IL-12/23 therapy, when patients undergo remission after 40 weeks of therapy, the treatment is terminated. When skin lesions reappear, patients are re-qualified for ustekinumab treatment (subgroup II and III). The number of patients suffering from psoriasis in I–III subgroups was associated with lasting remission, non-compliance of patients with recommendations and acute exacerbations, which resulted in the discontinuation of ustekinumab therapy (Fig. 1).

Patients classified for the study received ustekinumab in the doses dependent on their body weight (45 mg or 90 mg) according to the following scheme: 0–4–12 weeks. The control group consisted of 20 healthy volunteers (11 women and 9 men, aged 46 ±10 years) who gave their voluntary informed consent for the study.

Patients from the analyzed group were clinically examined on the day of therapy initiation, 4 weeks after they received the 1st medicine dose, and then every 12 weeks thereafter. The molecular analysis was performed parallelly to the clinical analysis.

The molecular analysis involved, first of all, an extraction of total RNA with the use of Fenozol (A&A

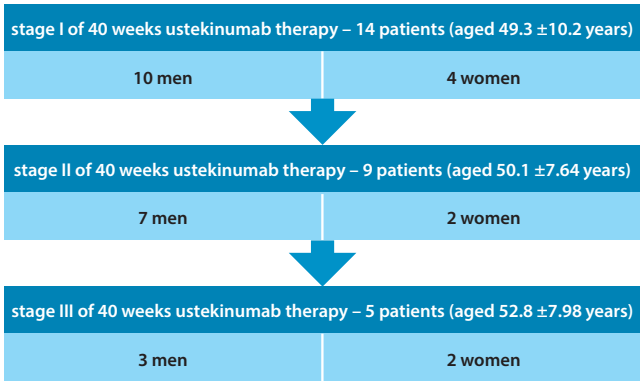

Fig. 1. Diagram showing which patients were involved in the subsequent phases of the study

Biotechnology, Gdańsk, Poland), according to the producer's recommendation. The next stage was connected with the amplification of genes (*TNF-α*, *TNFR1* and *TNFR2*) in the presence of β-actin, as an endogenic control, based on the quantitative reverse-transcription PCR reaction (RT-qPCR). The reaction was conducted with the use of a pair of starters, specific for the sequences of each analyzed gene and the use of SYBR Green Quantitect RT-PCR Reagent Kit (Qiagen, Valencia, USA), according to producer's recommendation.

The clinical analysis of the response to treatment was performed with the use of the following scales: PASI, DLQI and BSA. The statistical analysis was performed with the licensed version of STATISTICA v. 12.0 PL (StatSoft, Inc., Tulsa, USA) and included the Shapiro–Wilk test, the Mann–Whitney U test, the Friedman's analysis of variance (ANOVA), and the Spearman correlation. The statistical analysis was performed assuming the statistical gravity factor $p < 0.05$.

Results

The changes in the transcription activity of the genes analyzed during one stage of therapy using ustekinumab and in the control group were presented in the form of the quantity of mRNA copies of a given gene, converted into 1 μg of total RNA (Table 1).

Table 1. *TNF-α*, *TNFR1* and *TNFR2* expression profiles in patients with psoriasis vulgaris treated with ustekinumab and the control group (number of mRNA copies per 1 μg RNA)

Stage of therapy	Time [weeks of therapy]	Before/ after	*TNF-α*			*TNFR1*			*TNFR2*		
			median	lower quartile	upper quartile	median	lower quartile	upper quartile	median	lower quartile	upper quartile
Control	–	–	600000	300000	1000000	200000	60000	500000	300	40	1000
I	0	before	3407975	499749	7662592	366589	59986	1986452	71	15	213
	0	after	6027608	321856	49222471	2249138	815748	6864411	205	34	767
	4	before	10908550	3161565	44516325	561627	155839	2184666	74	16	278
	4	after	5733638	5292815	26745073	2117022	64335	3881999	318	59	1943
	16	before	17453616	8902661	31385633	525608	55374	1626370	168	26	4545
	16	after	25275607	14105295	35388972	1489566	118572	4818033	3334	74	8534
	28	before	1889572	29263771	107209432	947764	6400335	17378728	193	918	11057
	28	after	4293705	18846934	57529390	879275	7073464	45423569	285	782	16024
	40	before	1157602	5085281	14371656	352167	1744295	5167842	49	847	11286
	40	after	2500149	5564184	25853739	297111	362892	1735929	30	479	1653
II	0	before	883689	128266	1279348	417425	171418	901541	19	11	94
	0	after	1462641	892571	14678007	1539796	13251	9359111	814	107	152034
	4	before	171418	40210	3242518	359281	274	709177	147	33	199
	4	after	248168	33923	695135	475376	268837	475376	921	158	2480
	16	before	223566	11406	1006370	286526	19572	1090188	106	66	7088
	16	after	2056333	306159	4205319	219837	54823	1626370	100	30	927
	28	before	10481	1144	108213	9699	3721	54996	134	1	1178
	28	after	30533	621	50608	4826	3505	14792	5	1	21
	40	before	14363	505	39021	24220	57	50104	237	125	968
	40	after	256583	55930	1779532	978475	193273	1180987	116	13	252
III	0	before	143632	36749	307124	480153	26239	2094882	593	363	1444
	0	after	453617	89488	530652	285461	214	648139	54	0	82
	4	before	76257	43996	840591	1923	260	2571	3	0	5
	4	after	52673	10320	134842	14071	50	36981	3	0	4
	16	before	117354	47661	358023	4717	1383	103363	87	0	225
	16	after	1496530	274268	3697534	2252	17	19421	179	39	3463
	28	before	0	0	0	0	0	3	0	0	17
	28	after	0	0	10	0	0	0	126	0	12482
	40	before	0	0	0	0	0	0	596	8	1184
	40	after	0	0	0	0	0	0	0	0	0

As far as the molecular analysis is concerned, it may be observed that in the group under study, before therapy commencement, at stage I and II, the *TNF-α* expression was higher than that of the control group; however, it changed during stage III. *TNFR1* expression remained always higher in the study group when compared with the control group, while *TNFR2* is subject to overexpression when compared to the control, in the group of patients classified for stage III of therapy. A statistically significant difference in the *TNF-α* expression was observed between the group examined at stage I of therapy and the control group ($p = 0.011593$). We also ascertained the occurrence of a statistically significant transcription activity of *TNFR2* between the study group and the control group during the stage II of therapy ($p = 0.032511$). The *TNFR1* expression in the study group is higher than that of the control group, whereas an increased number of *TNFR1* transcript copies was observed at the reclassification.

A higher quantity of TNFR1 receptor copies compared to those of TNFR2 is observed in the study group as opposed to the control group.

The analysis of changes in the expression profile of the analyzed genes before administration of another dose of ustekinumab and 2 h after its administration enabled us to note that the *TNF-α* expression basically increased after medicine administration. A similar tendency was observed in the case of *TNFR1* and *TNFR2* receptors expression. However, during stage III of the classification for the anti-IL-12/23 therapy, it was observed that receptor expression decreased after medicine administration when compared with the activity before administration of the next medicine dose.

The characteristics of the clinical parameters (PASI, DLQI and BSA) of therapy efficacy are presented in Table 2. A gradual decrease of all 3 values of clinical parameters of the response to treatment during its duration could be observed. We also did not observe any increase in the values of the analyzed parameters between the subsequent inclusions to the anti-IL-12/23 therapy.

We established that the differences in the values of the individual clinical indices during a given therapy stage are statistically insignificant (stage I: PASI $p = 0.00000$; DLQI $p = 0.00022$; BSA $p = 0.00028$; stage II: PASI $p = 0.02733$; DLQI $p = 0.04773$; BSA $p = 0.03875$, stage III: PASI $p = 0.04206$; DLQI $p = 0.05756$; BSA $p = 0.24066$ NS).

We noted several statistically significant ($p < 0.05$) correlations (r) between the expression of the analyzed groups in a given moment of therapy and the clinical indices.

The correlation between DLQI and the expression of *TNFR1* ($r = 0.660578$) and *TNFR2* ($r = 0.642229$) may be observed during stage I in week 28. A correlation between *TNFR2* and *DLQI* ($r = 0.828571$) and *TNFR2* and BSA ($r = 0.828571$) is observed during stage II in the 4th week of anti-cytokine therapy. At the moment stage III of therapy was commenced (0 weeks), a statistically significant correlation was observed between *TNFR1* and DLQI ($r = -0.900000$) and *TNFR1* and BSA ($r = 0.900000$), *TNFR2* and BSA ($r = 0.900000$). After 4 weeks of stage III of the therapy, statistically significant correlations occured between *TNF-α* expression and BSA (–0.900000), as well as *TNFR1* expression and DLQI ($r = -0.894427$).

Table 2. Characteristics of the disease progress clinical parameters (PASI, DLQI and BSA) in patients with psoriasis vulgaris during the anti-IL-12/23 therapy (ustekinumab)

Stage of therapy	Time [weeks of therapy]	PASI			DLQI			BSA		
		median	lower quartile	upper quartile	median	lower quartile	upper quartile	median	lower quartile	upper quartile
I	0	24	27	15	21	23	16	60	65	28
	4	15	17	24	10	19	54	33	39	56
	16	8	11	14	6	10	17	23	44	55
	28	4	7	9	8	10	16	11	14	34
	40	3	6	9	7	8	10	6	9	22
II	0	20	14	23	20	18	24	42	39	53
	4	8	5	11	13	8	16	22	75	27
	16	3	1	6	10	5	12	8	3	27
	28	3	1	11	7	5	12	8	3	37
	40	3	1	4	6	3	9	6	4	9
III	0	13	12	33	22	18	23	32	27	49
	4	10	10	11	10	10	11	34	23	43
	16	5	3	7	10	6	10	13	8	18
	28	3	2	4	10	3	10	15	14	20
	40	3	3	4	5	4	5	6	6	7

PASI – Psoriasis Area and Severity Index; DLQI – Dermatology Life Quality Index; BSA – Body Surface Area.

Discussion

In this study, we assessed the efficacy of pharmacotherapy with the use of ustekinumab in patients with psoriasis vulgaris. The analysis was performed either based on the clinical indices of the disease progress stage (PASI, DLQI and BSA) or on a molecular analysis of the changes in the transcription activity of genes encoding TNF-α, TNFR1 and TNFR2. Fourteen patients with diagnosed psoriasis vulgaris were classified for the anti-IL-12/23 therapy. The said group was divided into 3 subgroups, as the patients needed to be reclassified to the biological treatment. This was caused by the occurrence and duration of the remission stage in some patients. The control group was composed of 20 healthy volunteers who gave their informed consent for the study. The whole period of the study within the framework of this work lasted 120 weeks, and the clinical and molecular analyses were performed during each stage of the treatment for 40 weeks.

The degrees of psoriasis advancement and the quality of life during ustekinumab therapy were assessed at the beginning of treatment, after 4 weeks and then every 12 weeks, which corresponded to the medicine administration schedule. The PASI, BSA and DLQI indices were used to assess the clinical condition of the patients. In order to efficiently assess the efficacy of the treatment, it is extremely necessary to use several scales of disease progress.[23–25]

Regarding the values of all 3 mentioned scales, a gradual decrease of their numerical values can be observed with the time of ustekinumab administration. This indicates that the applied anti-cytokine therapy is effective. Moreover, at the moment a given subsequent stage of anti-IL-12/23 treatment was commenced, lower values of PASI and BSA were observed when compared to the previous stages (PASI: 24 > 20 > 13; BSA: 60 > 42 > 32), and similarly in case of DLQI (21 > 20 < 22). This would indicate that, despite the necessity to reclassify the patients for subsequent pharmacotherapy, their physical condition was better than before commencing the earlier stage of the anti-IL-12/23 medicine administration. This could suggest a long-term reduction of the inflammatory process. We were able to ascertain during our observation that there was a significant clinical improvement in the 40th week of the treatment (PASI = 3; BSA = 6; DLQI: 7 > 6 > 5), regardless of the therapy stage. Our observations show that the biggest difference between the values of PASI and BSA during stage I and II of the therapy was observed as soon as after 4 weeks, and the greatest decrease of the values of ratios was noted between 4 and 16 weeks after the treatment. Consequently, taking into account the whole period of the study, it may be stated that the efficacy of the anti-IL-12/23 therapy is actually not lower with regards to the specific stage of the therapy,[1,8,13] and the period of clinical improvement seems to be extended.

The efficacy of the anti-IL-12/23 therapy was also ascertained with regards to patients with psoriatic arthritis,[26,27] Crohn's disease[28] and inflammatory bowel disease.[29]

It is extremely important to take into account the DLQI parameter to assess the efficacy of the applied pharmacotherapy, since it enables us to assume a holistic approach towards psoriatic patients. This makes it possible to observe the manner in which the treatment affects patients' self-esteem, performance of social roles, self-acceptance, and their reception by the social environment.[22,30,31]

The 2nd part of the work involved the possibility of using *TNF-α, TNFR1* and *TNFR2* expression as supplementary molecular markers to diagnose psoriasis vulgaris and assess the efficacy of the anti-IL-12/23 therapy. To this end, on the basis of the RT-qPCR methods, we evaluated the number of mRNAs of the gene in 1 μg of total RNA before administering the next dose of the medicine and 2 h after its administration. Personalization of medicine and molecular biological tools make it possible to increase both the number and safety of personalized therapeutic strategies.[10,32–34]

It may be observed that in the group under study, before the initiation of therapy, at stage I and II, the *TNF-α* expression was higher than in the control group; however, it changed during stage III. The transcription activity of *TNF-α* during the first 2 stages of the treatment is compliant with the observations made by other researchers.[35] The *TNFR1* expression remained always higher in the study group when compared with the control group, while *TNFR2* is subject to overexpression when compared to the control, in the group of patients classified for stage III of therapy. The statistical analysis of these 2 groups showed 2 statistically important differences – for the *TNF-α* ($p = 0.011593$) and *TNFR2* ($p = 0.032511$) transcription profiles. We noted the changes in the expression profiles of the analyzed genes during our observations; however, they proved to be statistically insignificant ($p > 0.05$).

Moreover, changes in gene expressions are observed as early as after 2 h following medicine administration, which suggests its quick action and the possibility to observe the molecular changes.[36,37]

Olczyk-Kwiecień et al. indicated the possibility and usefulness of using changes in the *TNF-α* expression as a supplementary molecular marker in patients with rheumatoid arthritis (RA).[37] Additionally, Komatsu et al. on the basis of their own observations emphasized the possibility to use TNF-α to determine the degree of lesion advancement in inflammatory bowel diseases,[38] cardiovascular diseases and cancers.[39,40]

Our studies also suggest that it is possible to use the analysis of the expression profiles of the examined transcripts as supplementary molecular markers of the efficacy of psoriasis therapy. Reasons for this include the occurrence of statistically significant correlations between expression of the analyzed genes and the clinical indices of disease advancement. The possibility to combine the clinical and molecular parameters in diagnosing and assessing psoriasis is also confirmed by observations made by other

research groups. They also observed higher levels of cytokine, which was analyzed by us in the group of psoriatic patients compared with healthy persons.[35]

The molecular mechanism of ustekinumab section is connected with its binding of the p40 subunit, common for IL-12 and IL-23, thus preventing the interleukins from interacting with the receptors. Consequently, the signaling pathways activated by IL-12 and IL-23 are suppressed. Tumor necrosis factor α constitutes the main, final product of the JAK STAT signaling pathway, which is activated by IL-12 and IL-23. Consequently, we should expect lower expression of the analyzed gene under the influence of anti-IL-12/23 therapy. We observed the complete suppression of the gene in the group of patients at the end of stage III of the treatment. The observed expression of the analyzed cytokine and its receptors, even during the anti-IL-12/23 therapy, may result from the mechanism of ustekinumab action. Ustekinumab hinders the bioactivity of IL-12 and IL-23, preventing the p40 subunit from binding with IL-12Rβ1 receptor, which is found at the surface of the immune system cells. For that reason, ustekinumab is not able to bind with IL-12/23 which have already interacted with IL-12Rβ1.[1,13] The continuous expression of *TNF-α* may indicate the presence of a certain pool of IL-12/23 that is not naturalized by ustekinumab. At the same time, the signaling pathways leading to TNF-α secretion are activated. Such observations are supported by the values of clinical parameters during pharmacotherapy, indicating an improvement. It must be noted that TNF-α shows activity that intensifies IL-12/23 secretion,[17] leading to permanent inflammation.[18–20]

Conclusions

The obtained results of the clinical and molecular analyses in patients with psoriasis vulgaris during the anti-IL-12/23 therapy indicate the efficacy of ustekinumab. It also seems that *TNF-α* could serve as a supplementary molecular marker in the diagnostics of psoriasis and the evaluation of the efficacy and safety of anti-cytokine therapy. In light of our observations, anti-cytokine therapy, oriented on molecular objectives, may constitute an effective form of therapy in cases of psoriasis vulgaris.

When evaluating the pharmacotherapy results, one should take into account the scale of disease clinical progression and the changes in the expression profiles of the analyzed genes.

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CIMT does not identify early vascular changes in childhood acute lymphoblastic leukemia survivors

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Abstract

Background. Childhood acute lymphoblastic leukemia (ALL) survivors are at an increased risk of cardiovascular disease development. It is believed that in the general population, this risk can be predicted with carotid intima-media thickness (CIMT) measurement.

Objectives. The objective of this study was to assess CIMT and to investigate the effects of blood pressure (BP) and lipid profile values on CIMT in childhood ALL survivors.

Material and methods. The study group comprised 81 childhood ALL survivors aged 5–25 years. The control group consisted of 52 age- and sex-comparable healthy children. Carotid intima-media thickness measurement, 24-hour BP monitoring and lipid profiles were evaluated in patients and controls.

Results. Despite significantly higher proportion of subjects with arterial hypertension (AH) (30/81 vs 10/52; $p = 0.0315$), the mean values of CIMT were not statistically different in childhood ALL survivors as compared to controls (0.4303 ± 0.03 vs 0.4291 ± 0.03; $p = 0.81$ and 1.096 ± 0.74 vs 1.027 ± 0.55; $p = 0.56$, respectively). Carotid intima-media thickness values were not statistically higher in ALL survivors with AH as compared to ALL survivors with normal BP (0.433 ± 0.03 vs 0.428 ± 0.03; $p = 0.82$). A significant positive correlation between 24-hour systolic BP standard deviation score (SDS) and CIMT-SDS in childhood ALL survivors was found ($r = 0.29$, $p = 0.009$), whereas such correlation was not observed in healthy controls ($r = 0.12$, $p = 0.39$). A significant correlation between z-score body mass index (BMI) and CIMT was found in controls ($r = 0.29$, $p = 0.031$) but not in childhood ALL survivors ($r = -0.05$, $p = 0.64$). No significant correlations between CIMT and other measured variables were found. Carotid intima-media thickness did not significantly correlate with time since ALL diagnosis ($r = 0.09$, $p = 0.39$).

Conclusions. Carotid intima-media thickness measurement shows limited feasibility and diagnostic accuracy for early assessment of vascular alteration in childhood ALL survivors. Other tests are needed to predict cardiovascular risk in childhood ALL survivors at the early stage of the follow-up.

Key words: children, cardiovascular risk, arterial hypertension, acute lymphoblastic leukemia, carotid intima-media thickness

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Introduction

Carotid intima-media thickness (CIMT) is a recognized marker of endothelial dysfunction. Carotid intima-media thickness measurement has become a useful non-invasive method for cardiovascular risk assessment not only for adults but also for children.[1] Measurement of CIMT has established its utility as an important tool for evaluation of subclinical atherosclerosis.[1] Increased CIMT was found in children with metabolic syndrome, congenital adrenal hyperplasia, obesity, type 1 diabetes mellitus, and also in childhood cancer survivors.[2–7] However, the lack of pediatric, population-based reference values makes CIMT not a widely accepted marker of subclinical atherosclerosis in youth with cardiovascular risk factors.[8]

The developing cardiovascular system in children is highly susceptible to the toxic effects of chemotherapeutic agents and their administration may cause endothelial damage leading to endothelial dysfunction.[9–11] It is believed that vascular alteration related to endothelial dysfunction leads to loss of vasodilatory capability and atherosclerosis.[12]

Advances in multimodal chemotherapy and supportive care have substantially improved long-term survival in children with acute lymphoblastic leukemia (ALL).[13,14] However, an increased number of childhood ALL survivors is accompanied by increased prevalence of cardiovascular disease (including metabolic syndrome and coronary artery disease) in adults who survived childhood ALL.[15,16] Disorders related to endothelial derangement are initiated early in life since atherosclerosis has its background in childhood.[1,17] Little is known about this process in children after ALL treatment. Some published reports have confirmed endothelial dysfunction by showing lower flow-mediated vasodilatation (FMD) but not increased CIMT in childhood ALL survivors as compared to controls.[18,19]

The aim of our study was to assess the CIMT and to investigate the effects of blood pressure (BP) and lipid profile values on CIMT in childhood ALL survivors and healthy controls.

Material and methods

Patients and controls

Between 1999 and 2012, 168 patients received intensive chemotherapy for ALL in our institution. All patients who survived in first remission and were older than 5 years were invited to participate in this study. Eighty-one patients (32 girls and 49 boys, aged 5–25 years) positively responded and were included into the study group. The control group comprised of 52 children (22 girls and 30 boys, aged 5–17 years) without any known chronic or severe medical condition. Informed consent was obtained from all parents/legal guardians and all children. The study was approved by the Bioethics Committee of Pomeranian Medical University in Szczecin, Poland (approval No. KB-0012/69/12).

Carotid intima-media thickness assessments

Ultrasonographic studies of the common carotid arteries (CCA) were performed using ultrasound system Philips iU22 (Philips Ultrasound, Bothell, USA) with a 3–9 MHz linear array transducer. Carotid intima-media thickness was measured according to the criteria of the Mannheim Consensus.[20] Depth of focus was set at 30 mm, frame rate of 25 Hz and gain of 60 dB. The arterial wall was assessed in a longitudinal B-mode view, strictly perpendicular to the ultrasound beam. Carotid intima-media thickness was measured at a distance of 10 mm proximal of the bifurcation and was defined as the distance from the border between the echolucent vessel lumen and the echogenic intima to the border between the echolucent media and echogenic adventitia. A semiautomatic edge detection system was used. All ultrasonographic studies were carried out twice by 2 independent radiologists. Carotid intima-media thickness values from the right and left side were recorded with the Philips iU22 software. Average CIMT values were subsequently recalculated and expressed as CIMT-standard deviation score (CIMT-SDS). This was computed according to the least mean squares (LMS) formula for SDS analysis: $SDS = [(Y/M(t))^{L(t)} - 1]/(L(t) \times S(t))$ where Y is the child's mean CIMT [mm], M(t) is median of Y, L(t) is the measure of skewness, and S(t) is the coefficient of variation. The LMS formula and reference values for the CIMT measurements were extracted from the data published by Doyon et al.[21]

Definition and diagnosis of arterial hypertension

Blood pressure assessments were performed with the use of a standard oscillometric 24-hour ambulatory blood pressure monitoring (ABPM) device (HolCARD CR-07; Aspel S.A., Zabierzów, Poland). Measurements were taken every 20 min during daytime (08:00–22:00) and every 30 min during nighttime (22:00–08:00). Blood pressure values were recorded all day (daytime and nighttime, respectively) as systolic (SBP), diastolic (DBP) and mean arterial blood pressure (MAP). Only readings of SBP < 240 mm Hg and >70 mm Hg, and DBP < 140 mm Hg and >40 mm Hg were considered valid. Arterial hypertension (AH) was defined as either mean SBP and/or mean DBP ≥ 95th percentile for age, height and sex during any recorded (daytime and/or nighttime) period.

All mean ABPM values (SBP, DBP and MAP) were recalculated using the LMS formula and expressed as 24-hour SBP-SDS, DBP-SDS and MAP-SDS. The LMS formula and reference values for the ABPM measurements were extracted from the data published by Wühl et al.[22]

Clinical and biochemical evaluation

All patients and subjects from the control group underwent a complete physical examination with assessment

Table 1. Characteristics of study patients and controls

Parameters	ALL (n = 81)	Controls (n = 52)	p-value
Mean age [years], median	12.1 ±4.6 (12)	11.7 ±4.2 (12)	0.63
Sex, female/male	32/49	22/30	0.59
Mean age at ALL diagnosis [years], median	6.6 ±4.27 (6)	NA	–
Mean follow-up time [years], median	5.7 ±3.96 (5)	NA	–
Follow-up time ≤5 years, number of patients (%)	36 (44.44)	NA	–
Follow-up time >5 years, number of patients (%)	45 (55.56)	NA	–
AH, number of patients (%)	30 (37)	10 (19.2)	0.0315
24-hour SBP, mean	114.6 ±9.2	113.7 ±7.6	0.53
24-hour DBP, mean	65.8 ±7.5	64.0 ±5.7	0.14
24-hour MAP, mean	89.5 ±7.5	88.2 ±5.7	0.28
24-hour SBP-SDS	0.336 ±1.26	0.284 ±1.05	0.81
24-hour DBP-SDS	−0.26 ±1.28	−0.569 ±1.09	0.15
24-hour MAP-SDS	1.415 ±1.31	1.205 ±0.96	0.32
BMI [kg/m^2]	19.6 ±3.99	18.9 ±4.39	0.41
BMI, z-score	0.44 ±1.03	−0.10 ±1.40	0.01
BMI-for-age (≥90 percentile), number of patients (%)	20 (24.7)	7 (13.5)	0.13
BMI-for-age (≥95 percentile), number of patients (%)	10 (12.3)	3 (5.8)	0.34
Total cholesterol [mg/dL]	162.9 ±34.6	144.7 ±33.2	0.003
Triglycerides [mg/dL]	96.8 ±71.2	80.7 ±46.9	0.15
LDL [mg/dL]	92.0 ±28.6	85.1 ±26.9	0.17
HDL [mg/dL]	60.5 ±14.9	51.4 ±16.1	0.001

ALL – childhood acute lymphoblastic leukemia survivors; AH – arterial hypertension; BMI – body mass index; SBP – systolic blood pressure; DBP – diastolic blood pressure; MAP – mean arterial pressure; SDS – standard deviation score; NA – not applicable; LDL – low-density lipoprotein; HDL – high-density lipoprotein.

of weight and height. Body mass index (BMI) as well as z-score BMI were subsequently calculated. Fasting blood samples were collected from all study subjects for serum total cholesterol, triglycerides, low-density lipoprotein (LDL), and high-density lipoprotein (HDL) measurements.

Statistical analysis

The data was expressed as mean ± standard deviation (SD). Continuous variables between groups (age, age at diagnosis, time of follow up, z-score BMI values, and SDS values) were compared using the two-tailed Mann–Whitney test. Fisher's exact test was used to compare unpaired, nominal variables. Pearson's coefficients or Spearman's coefficients were used to estimate correlations between longitudinal data. P-values ≤0.05 were considered significant.

The statistical analysis was performed using STATISTICA v. 13 (StatSoft, Inc., Tulsa, USA) software.

Results

Patient and control characteristics are presented in Table 1.

Mean age at evaluation of CIMT was 12.1 years and 11.7 years in the ALL and control cohort, respectively. The number of patients with AH was significantly higher in the ALL cohort (30/81) as compared to controls (10/52) (p = 0.0315; odds ratio (OR) = 2.47; 95% confidence interval (95% CI) = 1.08–5.63). Body mass index was not statistically different in the study groups; however, z-score BMI was statistically higher in the ALL cohort. Numbers of patients with BMI-for-age above or equal to 90th and 95th percentile were not different in both cohorts.

It was found that right CIMT, left CIMT and average CIMT as well as CIMT-SDS were not statistically different between childhood ALL survivors and the control group. This data is presented in Table 2.

Right CIMT, left CIMT and average CIMT as well as CIMT-SDS were found not to be statistically higher in childhood ALL survivors and controls with AH

Table 2. Comparison of CIMT in childhood ALL survivors and controls

Variables	ALL (n = 81)	Controls (n = 52)	p-value
CIMT R [mm]	0.4305 ±0.03	0.4297 ±0.03	0.89
CIMT L [mm]	0.4300 ±0.03	0.4285 ±0.03	0.78
CIMT average [mm]	0.4303 ±0.03	0.4291 ±0.03	0.81
CIMT-SDS	1.096 ±0.74	1.027 ±0.55	0.56

ALL – childhood acute lymphoblastic leukemia survivors; CIMT – carotid intima-media thickness; L – left; R – right; SDS – standard deviation score.

Table 3. Comparison of CIMT in childhood ALL survivors and controls with AH and with normal blood pressure

Variables	ALL AH(+) (n = 30)	ALL AH(–) (n = 51)	p-value	Controls AH(+) (n = 10)	Controls AH (–) (n = 42)	p-value	*p-value
CIMT R [mm]	0.435 ±0.04	0.428 ±0.04	0.78	0.431 ±0.04	0.429 ±0.04	0.89	0.89
CIMT L [mm]	0.431 ±0.03	0.429 ±0.03	0.89	0.426 ±0.04	0.429 ±0.03	0.78	0.78
CIMT average [mm]	0.433 ±0.03	0.428 ±0.03	0.82	0.428 ±0.03	0.429 ±0.02	0.93	0.93
CIMT-SDS	1.291 ±0.91	0.981 ±0.61	0.07	1.034 ±0.49	1.024 ±0.57	0.95	0.95

ALL – childhood ALL survivors; AH (+) – arterial hypertension; AH (–) – normal blood pressure; CIMT – carotid intima-media thickness; L – left; R – right; SDS – standard deviation score; *p-values for comparison of CIMT in childhood ALL survivors with AH and normal blood pressure controls.

Table 4. Comparison of CIMT, blood pressure values as well as lipid profile in childhood ALL survivors in relation to the follow-up time

Variables	Follow-up ≤5 years (n = 45)	Follow-up >5 years (n = 36)	p-value
CIMT R [mm]	0.4276 ±0.03	0.4342 ±0.04	0.39
CIMT L [mm]	0.4248 ±0.03	0.4365 ±0.04	0.10
CIMT average [mm]	0.4262 ±0.03	0.4353 ±0.03	0.18
CIMT-SDS	1.167 ±0.78	1.00 ±0.67	0.34
24-hour SBP, mean	113.4 ±9.9	116.2 ±8.0	0.17
24-hour DBP, mean	65.9 ±8.8	65.7 ±5.5	0.91
24-hour MAP, mean	88.7 ±8.5	90.6 ±5.9	0.26
24-hour SBP, SDS	0.545 ±1.29	0.073 ±1.18	0.09
24-hour DBP, SDS	–0.193 ±1.43	–0.345 ±1.07	0.60
24-hour MAP, SDS	1.451 ±1.41	1.369 ±1.19	0.78
Total cholesterol [mg/dL]	161.9 ±34.6	164.2 ±33.2	0.76
Triglycerides [mg/dL]	102.5 ±71.2	89.6 ±46.9	0.42
LDL [mg/dL]	91.0 ±28.6	93.3 ±26.9	0.72
HDL [mg/dL]	58.3 ±14.9	63.2 ±16.1	0.14

CIMT – carotid intima-media thickness; L – left; R – right; SBP – systolic blood pressure; DBP – diastolic blood pressure; MAP – mean arterial pressure; SDS – standard deviation score.

as compared to cohorts with normal BP. Carotid intima-media thickness values were found not to be statistically higher in childhood ALL survivors with AH when compared only to normal BP controls. This data is presented in Table 3.

Numbers of subjects with high (≥1 SDS) and low (<1 SDS) CIMT were not different in childhood ALL survivors group compared to control group (37/81 vs 24/52 and 44/81 vs 28/52; RR = 0.901, 95% CI = 0.755–1.305 and RR = 1.008, 95% CI = 0.766–1.325, p = 1, respectively).

There were no significant differences in CIMT and BP values as well as lipid concentrations with respect to the follow-up in childhood ALL survivors. This data is presented in Table 4.

A significant positive correlation between CIMT [mm] and z-score BMI in the control group only was found (r = 0.299, p = 0.031). A significant positive correlation between CIMT-SDS and systolic BP-SDS was found exclusively in the ALL cohort (r = 0.29, p = 0.009). We also found a significant positive correlation between z-score BMI and 24-hour SBP in the ALL cohort as well as in controls (r = 0.23, p = 0.034 and r = 0.29, p = 0.031, respectively). No significant correlations between CIMT [mm] or CIMT-SDS and the other measured variables (including mean 24-hour BP as well as total cholesterol, triglycerides, and LDL and HDL concentrations) were found. Furthermore, no significant correlations between CIMT [mm]/CIMT-SDS and the follow-up time in the ALL cohort were found (r = 0.09, p = 0.39 and r = 0.122, p = 0.28). The correlations between CIMT and all variables measured are presented in Table 5.

The correlation between 24-hour SBP-SDS and carotid intima-media thickness SDS (CIMT-SDS) in the ALL cohort is shown in Fig. 1.

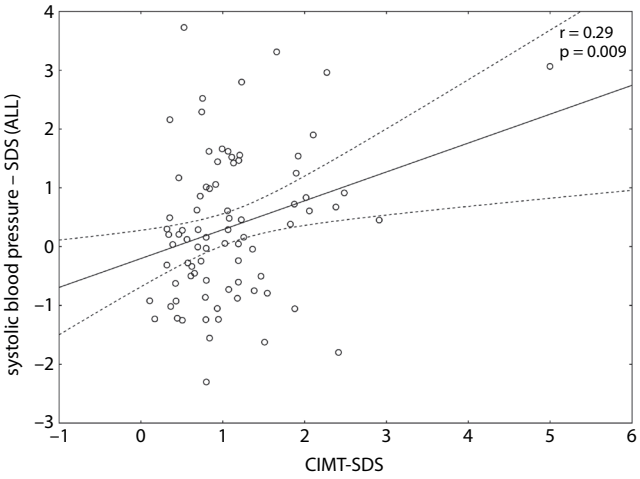

Fig. 1. The correlations between 24-hour systolic blood pressure (SBP-SDS) and carotid intima-media thickness SDS (CIMT-SDS) in childhood ALL survivors

Discussion

Assessment of cardiovascular risk in a population of childhood ALL survivors is challenging. A good, sensitive and commonly available method of early evaluation of subclinical cardiovascular disease in ALL survivors is strongly warranted and CIMT measurement as a direct estimation of arterial thickening seems to be promising. This non-invasive technique is commonly accepted as a credible instrument for cardiovascular risk assessment in adults. However, its use in the pediatric population is most commonly reserved for scientific purposes since

Table 5. Correlations between CIMT [mm]/CIMT-SD, blood pressure values and lipid profile in childhood ALL survivors and controls

Variables	CIMT [mm]		CIMT-SDS	
	ALL	controls	ALL	controls
z-score BMI r p-value	−0.0532 0.64	0.2999 0.031*	0.0611 0.59	0.1598 0.25
24-hour SBP, mean r p-value	0.2096 0.06	0.1889 0.18	0.314 0.19	0.0236 0.87
24-hour DBP, mean r p-value	0.1298 0.25	0.0444 0.755	0.066 0.56	−0.0468 0.74
24-hour MAP, mean r p-value	0.2133 0.056	0.0348 0.81	0.1316 0.24	−0.1109 0.43
24-hour SBP-SDS R p-value	0.1731 0.12	−0.0828 0.56	0.2904 0.009*	0.1219 0.39
24-hour DBP-SDS R p-value	0.1358 0.23	−0.0228 0.87	0.1179 0.29	−0.0376 0.79
24-hour MAP-SDS r p-value	0.1655 0.14	−0.1411 0.318	0.1988 0.07	−0.0438 0.76
Total cholesterol [mg/dL] R p-value	0.0098 0.93	−0.0605 0.67	−0.0198 0.86	0.1314 0.35
Triglycerides [mg/dL] R p-value	0.1869 0.09	0.1455 0.303	0.0906 0.42	0.1545 0.27
LDL [mg/dL] R p-value	0.0063 0.956	−0.0087 0.95	−0.0028 0.98	0.1568 0.27
HDL [mg/dL] R p-value	0.0275 0.8	−0.1155 0.41	−0.0148 0.89	−0.0214 0.88
Follow up [years] r p-value	0.09 0.39	NA NA	0.122 0.28	NA NA

ALL – childhood acute lymphoblastic leukemia survivors; SBP – systolic blood pressure; DBP – diastolic blood pressure; MAP – mean arterial pressure; BMI – body mass index; SDS – standard deviation score; r – Pearson correlation coefficient; * statistically significant; NA – not applicable; LDL – low-density lipoprotein; HDL – high-density lipoprotein.

pediatric data regarding the usefulness of CIMT is still limited.[1]

It is believed that cancer survivors, including children treated for ALL, are at particular risk of AH development.[16,23,24] The main finding of the presented paper is that CIMT expressed as mean values [mm] and SDS is not statistically different in childhood ALL survivors as compared to controls and between those ALL survivors with AH and normal BP. This is surprising since there is a lot of published data which implies a strong association between CIMT and disorders associated with atherosclerosis development. Increased CIMT was found in children with type 1 diabetes mellitus, familial hypercholesterolemia, hypertension, and metabolic syndrome.[2,4,7,25,26] Children after therapy of ALL are also at substantial risk of cardiovascular disease development in adulthood.[15,16,27] It was also confirmed that cancer childhood survivors are approx. 8 times more likely to die due to cardiac-related events as compared to the general population.[28]

Our observations are in line with data published by Giordano et al. They demonstrated that children after ALL treatment had lower FMD of the brachial artery but not different CIMT as compared to healthy controls.[18] Moreover, the study of Järvelä et al. revealed similar results since they did not find a difference in CIMT between childhood ALL survivors aged 16–30 years as compared to controls, although it needs to be stressed that one of the main aims of their study was to compare the CIMT values before and after a 16-week home-based exercise program.[19]

It should also be highlighted that most available pediatric studies regarding CIMT included older children and compared the mean values of CIMT in healthy subjects with a population at risk (hypertension, obesity, metabolic syndrome, and dyslipidemia). Doyon et al. established reference charts which allow a comparison CIMT-SDS in children with chronic conditions and healthy controls.[21] In our study, all mean values of CIMT were computed using the LMS formula, which makes it possible to convert the measurements for specific characteristics of a child (sex, age, height).

The relationship between AH and CIMT has been confirmed in many studies.[29,30] This convincing association is not in line with our results. Despite an increased number of individuals with AH in childhood ALL survivors as compared to controls, the CIMT values in these 2 populations were not statistically different.

If CIMT represents subclinical and asymptomatic atherosclerotic vascular disease which is also associated with AH, the only explanation why CIMT is not increased in hypertensive childhood ALL survivors, as compared to controls, is that CIMT displays only morphological alterations of the vascular wall, whereas one of the earliest components of atherosclerosis is endothelial dysfunction.[21] It results from the predominance of a pro-vasoconstrictory state and, if sustained and not reversed, may finally progress to morphological changes of the vascular wall including plaque formation.[31]

Several functional changes of the vascular wall in childhood ALL survivors may be present earlier, leading to abnormal regulation of vascular tone, and are responsible for AH development.[32] Even though we found a significant positive correlation between CIMT-SDS and 24-hour SBP-SDS in the ALL cohort, such a correlation was not found in controls. This may indicate some causative impact of BP on CIMT in children with ALL.

Our data cannot support a strong relationship between BP and CIMT in the patients studied. It is in contrast to data reported by Dawson et al., who found that SBP was independently associated with CIMT in a population of children older than 11 years.[33] Kollias et al., who found

that BP was an independent determinant of increased CIMT in healthy children aged 8–18, made a similar observation. It is worth noting that the correlation coefficient observed in that study was low (r = 0.12), suggesting that CIMT is only partially predicted by BP.[8]

Carotid intima-media thickness is particularly influenced by a patient's age; thus, the time of first CIMT assessment may be crucial for proper adaptation of this method.[21] Our study population consisted of subjects with a median age of 12 years and a median time of CIMT analysis of 5 years from ALL diagnosis. It might be too early to find structural abnormalities in the arterial wall. However, we did not find any significant correlation between CIMT and time from diagnosis of ALL, which might be explained by the relatively short time of the follow-up in the study group. The length of time that could result in morphological remodeling of the vascular wall in childhood ALL survivors still needs to be estimated. This is in line with data published by Baroncini et al., who found no significant difference in CIMT in healthy children younger than 15 years and relatively constant values of CIMT in children younger than 10 years.[34] The hypothesis that our study population was examined too early also cannot be ruled out.

Dawson et al. confirmed a direct association of CIMT with total cholesterol and triglycerides in a healthy population of adolescents and young adults.[33] We did not find any significant correlations of CIMT (mm/SDS) with total cholesterol, triglycerides, and LDL and HDL concentrations in childhood ALL survivors as well as in controls, even though the concentration of total cholesterol was higher in the ALL cohort.

This finding supports the observation of Giordano et al., who also did not confirm such a correlation although concentrations of total cholesterol, triglycerides and LDL were higher, and HDL was lower in ALL survivors as compared to controls.[18] But the truth is also that we found higher HDL concentration in ALL survivors, which would have protective effect for early vascular changes in this population.

Vijayasarathi et al. showed that the only risk factor which had a negative significant correlation with CIMT was HDL concentration. In this study, total cholesterol, LDL and triglyceride concentrations were not significantly correlated with CIMT.[25]

Metabolic syndrome, obesity and hyperlipidemia are widely recognized causative factors of atherosclerosis in youths. Published data indicates their relation with intima-media thickening, making CIMT evaluation a useful tool for prediction of cardiovascular events in such patients.[1,25] We found that z-score BMI was significantly higher in childhood ALL survivors. However, a significant correlation between z-score BMI and CIMT was found exclusively in the controls. Moreover, higher z-score BMI did not result in carotid intima-media thickening in the ALL cohort. Mean BMI as well as numbers of patients with obesity were also not different between the 2 groups. This is in line with the results of a study by Juonala et al. They found that the strength of the associations between childhood risk factors (total cholesterol, triglycerides, BP, and BMI) and CIMT is dependent on childhood age and becomes evident only in children older than 8 years.[17]

We are aware that our study has several limitations. The study sample was relatively small as well as the follow-up too short. These factors probably have had an impact upon the results. Moreover, measurements of CIMT require extreme precision, since Wiegman et al. found that CIMT increases by only 0.001 mm per year in healthy controls.[35] Finally, since reference values of CIMT for children have not yet been set, matched-case control is required for every measurement, which is not feasible in clinical practice.

Considering the results presented in this paper as well as the abovementioned study limitations, we conclude that measurement of CIMT cannot be used as an early indicator of endothelial dysfunction in childhood ALL survivors. Based on the observations presented in this study, one may speculate that in childhood ALL survivors with AH, the vascular wall retains its elasticity and probably responds to vasodilators. If this is so, these agents should be considered as first-line treatment of AH in young ALL survivors.

Although there are some clinical practice guidelines which provide recommendations for cardiac toxicity screening of late effects in survivors of pediatric malignancies using echocardiography, other tests are needed to predict cardiovascular risk in childhood ALL survivors at the early stage of the follow-up, in particular those with AH.[36] This would probably help in designing early intervention programs intended to prevent, reduce or delay cardiovascular disease in childhood and adolescence.

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Polymorphisms of the *MTHFR* gene in mothers of children with trisomy 21 (Down syndrome) in a Polish population

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Abstract

Background. Down syndrome (DS) is the most frequent cause of intellectual disability. In 95% of cases, it is caused by simple trisomy of chromosome 21 resulting from nondisjunction of chromosomes in meiotic division. Currently, the molecular and cellular mechanisms responsible for the phenomenon of nondisjunction are unknown.

Objectives. To investigate the incidence of 5 single-nucleotide polymorphisms (SNPs) of the *MTHFR* gene in a population of Polish mothers who had given birth to children with trisomy 21 in comparison with a control group of women with healthy offspring.

Material and methods. The test material comprised venous blood collected from mothers who had given birth to a child with DS (study group, n = 130) as well as from women who had given birth to children without trisomy 21 (control group, n = 88). DNA was isolated using a kit manufactured by Qiagen. Amplification was carried out using a Qiagen Multiplex PCR Kit (Qiagen); genotyping was performed using SNaPshot Genotyping MasterMix (Applied Biosystems).

Results. No statistically significant differences were observed in the frequency of genotypes between the examined groups in terms of the polymorphisms of the *MTHFR* gene.

Conclusions. In the Polish population studied, no relationship was found between the occurrence of particular genotypes of the *MTHFR* gene, i.e., 677CT, 1298AC, rs3737964, rs4846048, and rs1994798, in women and the birth of children with trisomy 21. The results contradict the validity of research on polymorphisms of the *MTHFR* gene as potential predisposing factors for the occurrence of trisomy 21 in children.

Key words: *MTHFR*, methylenetetrahydrofolate reductase, Down syndrome, simple trisomy of chromosome 21, single nucleotide polymorphism

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Background

Down syndrome (DS), known as trisomy 21, occurs at an estimated frequency of about 1/650–700 live births and is the most frequent known cause of intellectual disability. The chromosomal aberration associated with DS depends on the presence of an additional chromosome 21, which, in most cases (95%), is caused by a simple trisomy resulting from the phenomenon of chromosome nondisjunction during meiotic division in the process of gametogenesis in one of the parents.[1] The additional chromosome 21, in 90–95% of cases, is of maternal origin; the defective segregation of chromosomes occurs mainly during meiosis I (80% of cases) and less frequently during meiosis II (20% of cases).[2–4]

Currently, the molecular and cellular mechanisms responsible for the phenomenon of nondisjunction are unknown, as are the potential factors which predispose to its occurrence. The only documented risk factor for giving birth to a child with trisomy 21 is the mother's age (when >35 years).[4,5] In 1999, for the first time, studies were published in which it was suggested that polymorphisms within genes coding for enzymes indispensable in folate metabolism might constitute risk factors for chromosomal diseases, including DS.[6]

The products of the metabolism of folates are indispensable compounds used in many cellular processes, primarily in the synthesis of nucleic acid precursors and the methylation of cellular components, mainly DNA. The folate cycle also enables the conversion of homocysteine to methionine. The key enzyme involved in this cycle is the enzyme methylenetetrahydrofolate reductase (MTHFR), which catalyzes the irreversible reduction of 5,10-methylenetetrahydrofolate (5,10-MTHF) to 5-methylenetetrahydrofolate (5-MTHF), a methyl-group donor for homocysteine remethylation and methionine resynthesis. The end result, S-adenosylmethionine (SAM), the product of continued methionine conversion, is the basic compound required for the methylation of nucleic acids. DNA methylation plays a key role in gene expression as well as in cell differentiation. It has been proven that single nucleotide polymorphisms (SNPs) within the *MTHFR* gene cause a reduction in the activity of this enzyme, resulting in increased levels of homocysteine and in hypomethylation of nucleic acids.[1,5–6]

In vivo studies have demonstrated the influence of epigenetic phenomena, mainly DNA hypomethylation, on the occurrence of instability or incorrect segregation of chromosomes, or of aneuploidy.[7,8] In vitro studies on cell cultures (plant and animal) have confirmed that insufficient DNA methylation may result in chromosomal instability as well as nondisjunction and aberrations.[9,10] Similarly, a deficiency or lack of folates in the diet may affect the occurrence of DNA chain breaks and defective gene expression. Thus, researchers have reached the conclusion that folate deficiencies and abnormal expression of the *MTHFR* gene may pose a risk of chromosomal nondisjunction during meiosis, and, at the same time, increase the risk of trisomy 21 in children.[1,5–7]

The polymorphisms of the SNP type within the *MTHFR* gene most often researched and described are rs1801133 (677CT) and rs1801131 (1298AC). The former is dependent on the transition of cytosine to thymine at position 677 in the 4th exon of the *MTHFR* gene. The result is the conversion of alanine to valine in the amino acid chain at position 222 of the protein. The enzyme variant coded in this way is thermolabile and is characterized by reduced activity.[11–13] The change in rs1801131 occurs within exon 7 and consists of the transversion of adenine to cytosine at position 1298 of mRNA; instead of glutamine, alanine is incorporated at position 429 of the amino acid chain. The regulatory domain binding SAM undergoes modification, resulting in a reduction in the activity of MTHFR.[12] The authors of the study deemed it justified to take an interest in 3 other polymorphisms within the *MTHFR* gene: rs3737964, rs4846048 and rs1994798. The 1st, rs3737964, is located in the promoter region (5′ near gene) of the *MTHFR* gene and consists of the conversion of guanine to adenine. Sequencing changes in this region may affect the expression of mRNA and the amount of protein (enzyme) produced. The flow of mRNA may also be influenced by the 2nd polymorphism, rs4846048, which is located in the 3'UTR regulatory region and consists of the conversion of adenine to guanine. The last of the studied SNPs, rs1994798, is located within the intron and involves the exchange of cytosine for thymine. Polymorphisms located in introns are probably capable of modifying the expression of other genes or may be linked with other polymorphisms.[14–16]

Objectives

The study presented here investigated the frequency of 5 SNP-type polymorphisms of the *MTHFR* gene, 677CT, 1298AC, rs3737964, rs4846048, and rs1994798, in mothers of children with trisomy 21 and in women giving birth to healthy children, with the aim of assessing the relationship between the variability of these polymorphisms and trisomy 21 in a Polish population.

Material and methods

Research material

The study group consisted of 130 samples of venous blood collected with the use of ethylenediaminetetraacetic acid (EDTA) from women who had given birth to a child with trisomy 21. Prior to the isolation of DNA, all samples were stored in a freezer at a temperature of −80°C. Approval No. KB20/2016 of the Bioethics Commission was granted for molecular tests, and collection of the material lasted from February 2016 to February 2017. All of the women involved expressed their informed consent for their participation in the study. The age of the women at the birth

of the children with DS ranged from 18 to 41 years, with a mean of 32 years.

The control group consisted of 88 samples of isolated DNA, stored at −20°C at the Molecular Techniques Unit of Wroclaw Medical University, Poland. The DNA was collected in 2010–2012, with approval No. KB-556/2008 of the Bioethics Commission. The samples were taken from women who had given birth to children without trisomy 21 and who gave their informed consent for the use of their genetic material in molecular research. The age of the women ranged from 25 to 78 years, with a mean of 52 years.

Genotyping

In the case of the study group, first DNA was isolated, using the QIAamp DNA Mini Kit, from Qiagen (Hilden, Germany), according to the manufacturer's instructions. The DNA thus isolated was stored at −20°C.

Genetic material was propagated in a multiplex amplification reaction using a Qiagen Multiplex PCR Plus Kit and specific primers in accordance with the manufacturer's instructions. The amplified fragments (5 µL) were purified of unused deoxynucleotides and excess primers with 1.5 µL of a digestion enzyme mixture: exonucleases I (Exonuclease I 2.7 U; Thermo Fisher Scientific, Waltham, USA) and alkaline phosphatases (FastAP Thermosensitive Alkaline Phosphatase, 1.4 U; Thermo Fisher Scientific), with digestion carried out for 60 min at 37°C and for 15 min at 80°C (for enzyme inactivation).

Detection of polymorphisms was performed by means of mini-sequencing using a SNaPshot Multiplex Kit (Applied Biosystems and Thermo Fisher Scientific) in accordance with the manufacturers' instructions. Mini-sequencing primers were designed to hybridize to DNA directly at the changed polymorphic site. The sequencing reaction products were also purified of unused dideoxynucleotides using 0.5 µL alkaline phosphatase (FastAP Thermosensitive Alkaline Phosphatase, 0.5 U; Thermo Fisher Scientific) for 30 min at 37°C and 15 min at 85°C.

Detection of mini-sequencing reaction products was carried out by means of capillary electrophoresis in the presence of Hi-Di Formamide (Applied Biosystems) with the addition of 10 µL of the internal standard GeneScan-120 LIZ Size Standard (Applied Biosystems). Prior to electrophoresis, the samples were denatured for 5 min at 95°C; then the plate was cooled on ice for 3 min. Thus prepared, the plate was placed in a 3130 Genetic Analyzer sequencer (Applied Biosystems) and analysis of the results was conducted using GeneMapper ID v. 3.2 (Thermo Fisher Scientific).

Methods of statistical elaboration

STATISTICA v. 13.1 (StatSoft, Inc., Tulsa, USA) was used to elaborate the statistical results. Calculations of the frequency of individual genotypes as well as of the incidence of wild and mutant alleles in both studied polymorphic sites in the study and control groups were conducted using the χ^2 test. This test was also used to calculate whether the distribution of genotypes obtained in the studied population deviated from the estimated distribution, based on the Hardy–Weinberg principle. A significance level of 0.05 was assumed.

Results

In the 1st stage of statistical analysis, the studied polymorphisms were checked in terms of maintenance of distributions of genotypes in the population consistent with the Hardy–Weinberg equilibrium (HWE). None of the polymorphisms deviated from HWE; for 677CT, $p = 0.9986$; 1298AC, $p = 0.9958$; rs3737967, $p = 0.9956$; rs4846048, $p = 0.9982$; and rs1994798, $p = 0.9991$. Based on the results of genotyping obtained for all 130 samples from the study group and 88 samples from the control group, the frequency of individual genotypes at both polymorphic sites was calculated.

In the case of 677CT, the highest percentage in both groups was obtained for CT heterozygotes: 44.5% in the study group and 60% in the control group. The homozygous CC genotype constituted 40% in the study group and 32% in the control group, while homozygotes representing the TT mutation constituted 15.5% and 8%, respectively. Statistical analysis of the frequency of polymorphism in the studied groups showed no statistically significant differences ($p = 0.0548$). The frequency distribution is presented in Fig. 1.

Polymorphism 1298AC was characterized by the following distribution of genotypes in the studied population: in the study group, the homozygous AA configuration accounted for 53%, the heterozygous AC for 37%, and the homozygous CC for 10%, whereas, in the control group, these genotypes accounted for 38.5%, 51% and 10.5% of the population, respectively. In comparing the frequency of the occurrence of genotypes in the studied groups, no statistically significant differences were found according to the χ^2 test ($p = 0.0905$). The distribution of genotypes for the 1298AC polymorphism is presented in Fig. 1.

At the site of rs3737964, the frequency of individual genotypes was as follows: the homozygous GG configuration constituted 55.5% in the study group and 40.5% in the control group; heterozygous GA – 35.5% and 49%, respectively; and homozygous AA – 9% and 10.5%. respectively. Comparison of these frequency distributions using the χ^2 test showed no statistically significant differences ($p = 0.0986$). These distributions are presented in Fig. 1.

At the polymorphic site of rs4846048, in both the study and control groups, the homozygous AA configuration constituted the largest percentage: 57% in the study group and 49% in the control group. AG heterozygotes were recorded at 35.5% and 40.5%, respectively, mutant GG

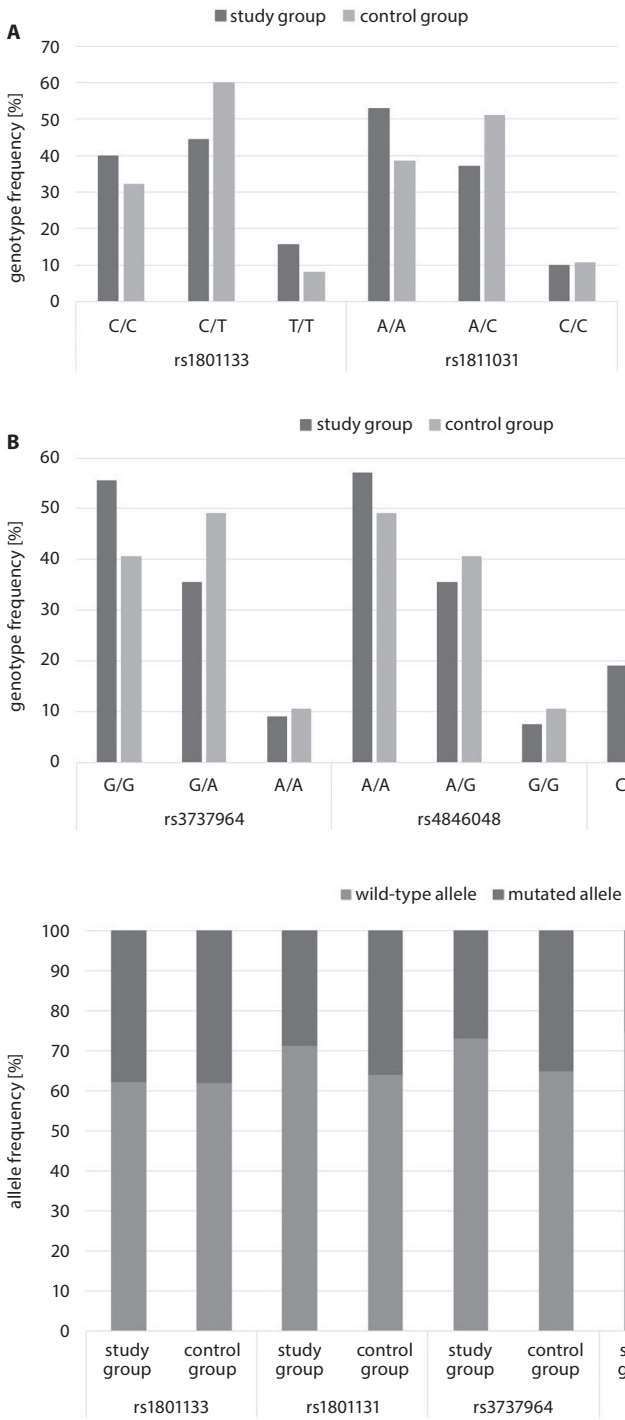

Fig. 1. Genotype frequencies of investigated SNPs in *MTHFR* gene in study group compared to control group. A – SNPs located in exons; B – SNPs located outside exons, in various functional regions of MTHFR gene

Fig. 2. Frequency of mutant alleles and wild-type alleles in 677CT and 1298AC, as well as rs3737964, rs4846048 and rs1994798, in the study and control groups. The results indicated no statistically significant differences ($p > 0.05$ in all cases)

heterozygotes at 7.5% and 10.5%, respectively. No statistically significant differences were indicated in the distribution of genotypes ($p = 0.4790$). The above data is presented in Fig. 1.

The following distribution of genotypes of the rs1994798 polymorphism was observed: for the homozygous CC configuration, 16% in the study group and 19.5% in the control group; for CT heterozygotes, 45.5% in the study group and 51.5% in the control group. In this case, as well, statistical analysis showed no statistically significant differences ($p = 0.3321$). The frequency distribution is presented in Fig. 1.

Analysis of the frequency of mutant and wild alleles in the control and study groups also showed an absence of statistically significant differences in the cases of both studied polymorphisms ($p > 0.05$). Wild alleles (C) at 677CT accounted for 62.5% in the study group and 62% in the control group, whereas mutant alleles (A) accounted for 37.7% and 38%, respectively ($p = 0.9552$). Similarly, in the case of 1298AC, wild alleles (A) accounted for 71.5% in the study group and 64% in the control group, while mutant alleles (C) for 28.5% in the study group and 36% in the control group ($p = 0.2524$). These frequencies are presented in Fig. 2.

Discussion

Trisomy 21 (also called DS) is a relatively common affliction, and the most widely known cause of intellectual disability, hence the investigation and determination of the molecular basis of this disease and the search for potential predisposing factors for its occurrence are justified. Simple trisomy, constituting 95% of cases, results from the phenomenon of nondisjunction which occurs during meiotic division; however, the mechanisms responsible for the abnormal segregation of chromosomes are still unknown.[2]

In the search for the causes of faulty segregation of chromosomes, their instability, fragility or aneuploidy, an ever greater attention is being drawn to the potential contribution of epigenetic phenomena, primarily DNA hypomethylation.[7,8] Deficiencies in dietary folate and disorders of the folate cycle which directly result in, i.a., hyperhomocysteinemia and insufficient synthesis of SAM – a fundamental donor of methyl groups necessary for DNA methylation – have been linked to the hypomethylation of nucleic acids. Disorders of the cyclic transformation of folate derivatives result primarily from a defect in the activity of the key enzyme in this cycle, i.e., MTHFR. Currently, more than 40 SNP polymorphisms have been described in the *MTHFR* gene; the direct influence of some of these on the reduction of the activity of the enzyme encoded by this gene, ultimately resulting in SAM deficiencies and DNA hypomethylation, has been proven.[1,5–7,11,17] On this basis, a conclusion was drawn regarding the potential for linking nondisjunction, the reason for the vast majority of DS cases, with DNA hypomethylation, which can be brought about by disturbances of the folate cycle resulting from insufficient MTHFR activity, caused in turn by the occurrence of polymorphisms of single nucleotides within the associated gene.

In this study, the 2 SNP polymorphisms most frequently associated with trisomy of chromosome 21 in *MTHFR* were taken into consideration, namely 677CT and 1298AC, as well as 3 others, not previously researched for the existence of correlations with DS, namely rs3737964, rs4846048 and rs1994798. Observed differences between the study and control groups in the frequency of genotypes in 677CT and 1298AC, as well as in all 3 remaining polymorphic sites, showed no statistical significance in the χ^2 test ($p > 0.05$). The distribution of the percentage of frequency of mutant and wild alleles in the studied SNPs also fell within the confidence interval in the χ^2 test ($p > 0.05$); thus, no statistically significant differences were observed in the studied groups in the Polish population.

The results presented in this paper are congruent with many other reports published in the relevant literature since the appearance of the first paper on this subject in 1999. This applies equally to studies of Caucasians and of other populations. Worth mentioning, i.a., are studies on French (Chango et al., 2005),[18] Italian (Stuppia et al., 2002),[19] Danish (Kokotas et al., 2009),[20] and Croatian (Vraneković et al., 2010)[21] populations. In addition, analogous studies which also excluded the association of DS with the SNP polymorphisms 677CT and 1298AC were carried out in Jordan (Sadiq et al., 2011),[22] Romania (Bucerzan et al., 2017),[6] Brazil (Balarin et al., 2017),[5] Turkey (Boduroglu et al., 2004),[23] China (Jiajin et al., 2018),[1] and India (Kohli et al., 2008; Kaur et al., 2013; Mohanty et al., 2012).[24–26] Confirmation was also obtained in an extensive meta-analysis by Yang et al. (2013), encompassing 32 articles excluding the association of *MTHFR* polymorphisms of 677CT and 1298AC with trisomy of chromosome 21.[27]

On the other hand, an equal number of researchers have proven a relationship between the occurrence of the described polymorphisms in the *MTHFR* gene in women and the birth of children with DS. This is evidenced by the first study by James et al. (1999) on an American population.[7] Similar results were obtained by Cyril et al. (2009, southern India),[28] Coppedè et al. (2009, Italy),[29] Meguid et al. (2008, Egypt),[30] and da Silva et al. (2005, Brazil).[31] Mention may also be made of meta-analyses conducted by Wu et al. (2012),[32] Rai et al. (2014)[33] and Victorino et al. (2014).[34]

The glaring discrepancies between these reports renders any inferences about single nucleotide polymorphisms 677CT and 1298AC as potential predisposing factors for the occurrence of DS in children doubtful and controversial, at the very least. The present paper also indicates the absence of any connection between the other 3 polymorphisms (rs3737964, rs4846048 and rs1994798) and the occurrence of DS. All of the abovementioned authors emphasize that these discrepancies exclude SNPs in the *MTHFR* gene as an independent risk factor for trisomy 21. The polymorphisms in question may nevertheless constitute one of many factors, including additional genetic, epigenetic, environmental, and other random factors, whose simultaneous interaction may result in a predisposition to the birth of a child with trisomy 21.[29]

The results of the present study of a Polish population do not contradict the reports published to date. It is not out of the question that larger sample size (in both the test and control groups) would result in statistically significant differences between the frequencies of individual genotypes of 677CT and 1298AC in the studied groups, given that the results of statistical analysis in some comparisons were close to decisive values. However, neither does the exclusion of a relationship between these polymorphisms in the *MTHFR* gene in mothers and the birth of children with trisomy 21 in this Polish population equate to the lack of participation of the described changes in the molecular basis of DS. The most appropriate direction of research appears to be an investigation of the simultaneous influence of several factors in the pathomechanism of DS; this would require additional, extremely detailed analyses.

Conclusions

In the present paper, no correlation was observed between the occurrence of polymorphisms in the *MTHFR* gene in mothers and the birth of children with DS in a Polish population. The results contradict the validity of research on the 677CT and 1298AC (as well as rs3737964, rs4846048, and rs1994798) polymorphisms of the *MTHFR* gene as potential predisposing factors for the occurrence of trisomy 21 in children.

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Pulmonary rehabilitation in interstitial lung diseases: A review of the literature

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Abstract

There are more than 200 different diseases classed as interstitial lung diseases (ILDs). For epidemiological and practical purposes, ILDs are classified into diseases of known and unknown etiology. The aim of this review is to evaluate our current knowledge about the efficacy and safety of pulmonary rehabilitation (PR) in patients with ILDs. Other issues, such as ILD pathogenesis, prevalence and comorbidity, are also elaborated in the review. Pulmonary rehabilitation is an important part of comprehensive care for patients with ILDs. In comparison to PR for patients with chronic pulmonary obstructive disease (COPD), the number of clinical studies concerning PR for patients with ILDs is small. The majority of trials have been performed in relatively small groups of patients. The principles of PR in this group of patients are the same as for patients with COPD. Exercise-induced desaturation is frequently observed during PR, which is the main source of complications in patients with ILDs. Major differences between ILD and COPD patients include poorer exercise tolerance and faster development of respiratory failure in patients with ILDs.

Key words: pulmonary rehabilitation, airway management, interstitial lung diseases

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The pathogenesis and prevalence of interstitial lung diseases

Interstitial lung diseases (ILDs) constitute a heterogeneous group of about 200 diseases characterized by acute or chronic follicular and bronchial inflammation and progressive, usually irreversible, pulmonary fibrosis, bilateral diffuse pulmonary lesions in imaging examinations, and ventilation restrictions. Despite the differentiated etiology, the clinical image of ILDs is similar, and results from the presence of diffuse and irreversible fibrous lesions of the alveolar parenchyma of the lung interstitium. Specific forms of ILDs can be differentiated from one another based on clinical data, radiological imaging and lung biopsy. The histopathologic changes in the lungs of patients with ILDs can range from granulomatous inflammation without parenchymal fibrosis in patients with sarcoidosis to expansive pulmonary fibrosis with architectural distortion of the lung in patients with idiopathic pulmonary fibrosis (IPF). Clinical ILD features include exercise-induced dyspnea, exercise-induced hypoxemia, progressive skeletal muscle weakness, and deterioration of exercise tolerance. The main symptoms of fibrosis are progressive decreases in forced vital capacity (FVC) and in the diffusing capacity of the lung for carbon monoxide (DLCO). Restriction of pulmonary ventilation leads to respiratory failure, further enhanced by diminished alveolar blood flow resulting from capillary destruction and hypoxic vasoconstriction.[1] The onset of the disease is usually non-specific; patients may complain of a low-grade fever or a fever with unclear etiology, coughing and deteriorated exercise tolerance. If the patient is a smoker, chronic pulmonary obstructive disease (COPD) is usually suspected, because spirometry focusses on disorders in lung ventilation. In patients with ILD, airflow in the respiratory tract is not disturbed; obstruction is rare, except in patients with sarcoidosis.[2] A diagnosis of ILD is sometimes based on changes in the chest X-ray image, especially in patients in whom this examination is performed periodically due to occupational exposure. In some cases, the onset of ILD may be acute, with symptoms requiring immediate medical intervention: pneumothorax, hemoptysis and rapid progressive respiratory failure. Interstitial lung diseases may be associated with exposure to occupational and environmental factors, drugs (amiodarone, chemotherapy drugs, methotrexate), or be accompanied by multiple connective tissue diseases (systemic lupus erythematosus, rheumatoid arthritis, systemic scleroderma, or dermatomyositis). In many cases, the etiology is unknown.

In a European study the prevalence of ILDs was estimated of 97.9/10,000. The most prevalent diagnoses were sarcoidosis (42.6%), connective tissue disease-associated ILDs (CTD-ILDs) (16%), IPF (11.6%), and occupational ILDs (5.0%).[3] The data from earlier studies also showed that the most frequent ILDs are IPF and sarcoidosis, which together comprise about 50%.[4]

Management

Due to the different pathogenic mechanisms, treatment for ILDs varies depending on the diagnosis. In the case of a known cause, such as for example exposure to environmental agents or drugs, the primary treatment is to eliminate the factors causing the disease. If the disease has an autoimmune background, glucocorticoids and immunosuppressive drugs are used to reduce the inflammation leading to progressive fibrosis. However, in some diseases of unknown etiology, such as IPF, there are still no effective pharmacological methods that significantly improve survival. Intensive research has been carried out in the past decade to find effective antifibrotic and anti-inflammatory drugs.[5]

Thus ILD treatment often includes only oxygen therapy in case of respiratory failure. The progression of lung fibrosis depends on the underlying disease, and is particularly rapid in IPF. In selected patients with treatment-resistant progressive ILD, the only therapeutic method is lung transplantation. However, due to an insufficient number of donors and numerous contraindications, access to this method is limited. Therapeutic options for ILDs are often limited, and do not guarantee improvement in the quality of life or its prolongation.[5,6] An increasing number of reports indicate that in most patients with ILDs, pulmonary rehabilitation (PR) is a widely accessible treatment that can significantly improve exercise capacity and reduce dyspnea.[7]

Pulmonary rehabilitation principles

Pulmonary rehabilitation is an evidence-based, multidisciplinary and comprehensive medical intervention dedicated to patients with a variety of symptoms of chronic diseases and reduced daily activity. The combination of PR with an individualized treatment plan reduces the symptoms, improves physical fitness, enhancing the functional and psychological status and quality of life of patients with respiratory diseases. The primary problem of patients with progressive chronic lung diseases is a gradual increase in symptoms such as dyspnea, persistent cough, easy fatigue and weakness, which cause restrictions of daily physical activity, resulting in muscle weakness and functional limitations. The patient becomes disabled, often experiences anxiety and/or depression and gradually withdraws from social life. The quality of life deteriorates considerably.

The purposes of PR include physical capacity improvement, reduction in disease symptoms, relief from negative emotional states, development of a healthy lifestyle with adequate physical activity, elimination of smoking, and giving patients the ability to self-monitor their symptoms, the course of the disease and their compliance with recommended treatments. Pulmonary rehabilitation includes physical examinations, chest physiotherapy, individual strength,

endurance and respiratory training, as well as relaxation techniques aimed at reducing muscle tension and anxiety and/or depression. Patients are educated about the disease, lifestyle, nutrition, and the harmfulness of smoking.

Pulmonary rehabilitation has been used for many years in patients with COPD, and numerous benefits have been noted, particularly reduced dyspnea, increased effort tolerance and improved quality of life.[7] The indications for PR and its benefits for patients with COPD have been well-defined.[8]

According to American Thoracic Society/European Respiratory Society guidelines, rehabilitation should be performed by a multidisciplinary team of pulmonologists, physiotherapists, psychologists, and nurses.[7] The first step is detailed diagnostic testing that allows the PR to be adjusted to the current state of the patient, including an evaluation of the patient's quality of life, the degree of anxiety and depression, and an evaluation of minimal exercise tolerance. In addition, the strength of the peripheral muscles can be assessed. A 6-minute walk test (6MWT) is a commonly used method of evaluating exercise tolerance. It is extremely important to evaluate O_2 saturation during exercise, and in particular the greatest decrease in O_2 saturation during the 6MWT (nadir SPO_2).[8]

Pulmonary rehabilitation includes both education and supervised exercise. The scope of the education should include an explanation of the pathomechanisms of the disease, an explanation of the appropriate treatment, breathing techniques, principles of healthy nutrition, indications for air travel in patients with respiratory failure, the correct use of inhalers, and dealing with stress. Physical exercises include individually modified endurance and aerobic training.

The main problems hindering the widespread use of PR are limited funding from healthcare systems despite increasing awareness of the benefits of PR, a lack of knowledge among physicians and a shortage of experienced rehabilitators. Pulmonary rehabilitation should be standard care in patients with COPD, along with pharmacotherapy, oxygen therapy and non-invasive ventilation.

Pulmonary rehabilitation favorably modifies the course of COPD in many ways, causing:

1. reduced number of hospitalizations,
2. increased exercise tolerance,
3. reduced dyspnea,
4. increased resistance and muscle strength,
5. improved quality of life,
6. reduced emotional disorders,
7. increased self-reliance,
8. increased fat-free body mass,
9. increased breath volume and oxygen saturation, and
10. improved capacity of the skeletal muscles at the cellular level.[7]

From a clinical point of view, the exercise component of PR increases the patient's physical capacity, which is the most often measured by the 6MWT or oxygen uptake during a maximal cardiopulmonary exercise test (maximal oxygen uptake – VO_2max). The obvious benefits of PR mean that over the years, attempts have been made to extend PR indications to other groups of patients with chronic lung diseases.

Pulmonary rehabilitation in ILDs

Although ILDs and COPD are completely different diseases, they are similar in many ways: Both diseases involve respiratory distress, increased respiratory effort and abnormal gas exchange, and the incidence of anxiety and depression is higher than in the general population. Numerous similarities indicate that PR in patients with ILDs may produce similar beneficial effects as in those with COPD.[9] Among ILD patients, those with IPF have particularly poor prognoses. The median of survival since the diagnosis has not changed in this group in the last 30 years, and it is no more than 5 years.[10] Until recently, apart from oxygen therapy, there was no therapeutic option for these patients. Because of the rapid progression of the disease, patients with IPF are rarely qualified for PR. The recent introduction of 2 new medicinal products for IPP – pirfenidone and the tyrosine kinase inhibitor nintedanib – may improve this situation. Early addition of PR to pharmacotherapy can improve the patients' survival.[6]

No guidelines concerning the optimization of exercise for patients with ILDs have been developed yet. Randomized trials have included all interstitial disorders or have been dedicated to a defined subgroup, such as patients with IPF.[11,12] The results of PR results are highly affected by the diagnosis. This especially applies to IPF patients, who show less improvement in physical performance after PR compared to patients with other ILDs. However, the faster course of IPF compared to most ILDs should be taken into account.[13]

The basic parameter for PR performance evaluation is the change in minimal important difference (MID) in the 6MWT. The MID has been set at 54 m in people with COPD, although there are opinions that this value is exaggerated.[14] For patients with ILDs, the MID value has not been established; the range varies from 24 m to 45 m, depending on the statistical method chosen.[15,16] The severity of symptoms, mainly dyspnea, is also evaluated. The Borg Dyspnea Scale and the Medical Research Council's (MRC) Baseline Dyspnea Index (BDI) and Transition Dyspnea Index (TDI) are used to evaluate the severity of dyspnea. Cough, depression, anxiety, and fatigue may also be evaluated. For the health-related quality of life evaluation, the most commonly used questionnaires are the Short Form Health Survey (SF-36), the Chronic Respiratory Disease Questionnaire (CRDQ) and St. George's Respiratory Questionnaire (SGRQ).[17,18] An additional parameter is pulse oximetry during exercise to determine nadir SpO_2 during the 6MWT.[19]

The number of controlled PR studies in ILDs is small, but a systematic increase is noticeable. A meta-analysis published in 2014 included a total of 8 papers concerning PR in patients with ILDs. No side effects were observed in any of the studies. The weighted mean difference in the distance in the 6MWT was 44.34 m. Statistically significant reductions in dyspnea was observed in all the studies. Improved quality of life was identified using various questionnaires. The improvement directly after PR that was observed in the subpopulation of IPF patients was similar to that of the whole population examined. The limitation was the small number of study groups (in total, 86 patients underwent PR and 82 comprised the control groups).[20] In a meta-analysis of 142 patients with IPF published in 2018, 4 studies evaluated the short-term benefits of PR. Significant improvement was found in the 6MWT, along with a significant decrease in the total SGRQ score.[21]

In a large study including 402 patients on a widespread ILD spectrum, PR was performed at a specialist center for an average of 30 days with 5 sessions per week. Clinically significant improvements in the patients' quality of life, physical capacity (the mean increase compared to the baseline 6MWT was 46 m) and a slight improvement in FVC (+1%) were observed. The only predictor of a significant response to PR was a low baseline 6MWT; the largest increase in the distance was observed in this group. Low 6MWT scores occurred in patients with low FVC, low total lung capacity (TLC) and treated with home oxygen therapy. In this study, conducted in an inpatient setting, 80% of the patients were on home oxygen therapy. This is further evidence of the value of PR use in patients with advanced respiratory failure.[22]

Different parameters are evaluated in different publications. The manner in which PR is performed (outpatient or inpatient) also varies from study to study; the study groups include patients with assorted ILDs or only 1 disease, e.g., IPF, and the duration of PR varies. However, despite the lack of detailed guidelines, PR is becoming an increasingly common component of multidisciplinary care for people with ILDs.

It is currently believed that patients with ILDs should be included in PR programs typically lasting 8 weeks, with at least 2 weekly sessions of at least 30 min of aerobic training. Spirometry tests play a lesser role in qualifying ILD patients for PR than in case of COPD, because a small degree of dysfunction in lung function tests is often observed in patients with significant limitations to exercise tolerance. The mechanism of exercise dyspnea in ILDs is complex. The primary role is played by dysfunction of the alveolar–capillary barrier associated with thickening of the pulmonary artery walls. Developing pulmonary hypertension is a result of chronic hypoxia-induced vasoconstriction and thromboembolic changes leading to the destruction of blood vessels. There is a strong correlation between increases in pulmonary pressure and decreases in exercise tolerance. In addition, an impaired chronotropic response of the heart to exercise and prolonged tachycardia are often observed, which may indicate cardiac sarcoidosis. In some patients, an excessively fast resting heart rate may limit exercise capacity, since the heart rate prevents the patients from continuing exercise shortly after they begin. In this situation, the use of drugs with negative chronotropic effects can be beneficial in terms of the results of rehabilitation. However, the implementation of this kind of therapy in the population with pulmonary pathologies has numerous limitations, including the small group of available drugs. On the one hand, some of them, such as ivabradine, have not been tested in these patients; and on the other, β-blockers may have a potentially adverse effect. Nebivolol, a β1-selective agent, is preferred in these patients because it causes quite low negative chronotropic effects at the standard dose used in hypertension compared to other representatives of the class. Another common problem is the coexistence of left ventricular diastolic dysfunction in many patients. In this situation, a lack of proper heart rate control impairs exercise tolerance, thus significantly affecting training opportunities.

Worsening tolerance to exercise may also be caused by skeletal muscle dysfunction. The mechanisms of this phenomenon include changes in skeletal muscles in the course of an underlying disease (e.g., dermatomyositis); myopathy induced by chronic systemic steroid therapy; and progressive immobilization of the patient.[22]

A progressive decrease in the 6MWT is a predictor of increased mortality, especially in IPF patients, among whom a drop in the 6MWT by more than 61 m in 6 months and desaturation below 88% during exercise are associated with increased mortality.[8]

The course and prognosis of ILDs is affected not only by the primary disease, but also by accompanying ones. Exercise tolerance in ILD patients is mainly affected by cardiovascular and metabolic diseases and smoking.[23] Accompanying diseases are divided into pulmonary and extrapulmonary ones. Pulmonary fibrosis enhances the expression of cytokines and growth factors, promoting the development of atherothrombosis through an increase in systemic inflammation and hypercoagulability. This leads to a two-fold increase in the risk of ischemic heart disease (IHD) compared to the general population. The coexistence of pulmonary fibrosis and IHD significantly shortens the patients' survival time.[20] Among IPF patients, as many as 1/3 have recognized IHD. The risk of venous thromboembolism is more than three-fold higher in the population of patients with ILDs compared to the general population. This requires early implementation of antithrombotic prophylaxis during hospitalization.[24]

Pulmonary arterial hypertension (PAH), defined as mean pulmonary arterial pressure ≥25 mm Hg evaluated during right heart catheterization, may occur in any chronic lung disease. This also applies to patients with ILDs, among whom the risk of PAH depends on the diagnosis.

It is generally accepted that the more severe the course of the disease is, the higher the incidence of PAH. For example, PAH is more common in patients with IPF than in cases of sarcoidosis. A diagnosis of PAH results in rapid deterioration of physical performance, the need for home oxygen therapy and increased mortality. Transthoracic echocardiography and right heart catheterization are routinely used in the diagnostics. The simple test is the 6MWT, which reveals an impaired normalization of the heart rate after exercise: A heart rate difference immediately after exercise and after 1 min rest that is lower than 13 bpm suggests PAH.[25] Special attention is required in case of patients with PAH in the phase of right-sided heart failure. The presence of clinically prominent PAH leads to recommendations for limiting intense exercise, with the fear that exercise-induced rapid pulmonary hypertension with the symptoms of right ventricular circulatory failure may lead to sudden death.[26] Pulmonary arterial hypertension patients are at an increased risk of PR complications, including arrhythmias, syncope and dizziness, which may occur in 13% of patients.[26,27]

Physical exercise is associated with sympathetic activation, which increases the risk of arrhythmias. At the same time, the population of patients with respiratory diseases is thought to have hypoxia-related arrhythmias. In the light of some research, this is not a significant impediment to physical activity. Although the majority of patients had 24-hour asymptomatic single premature supraventricular and ventricular beats, no significant arrhythmia was observed during the 6MWT despite a significant decrease in SpO_2.[27]

The risk of complications during PR decreases when interval training with a maximum heart rate increase up to 120 bpm and a combination of endurance and strength exercises is used.[28]

Gastroesophageal reflux disease (GERD) is a very common extrapulmonary comorbidity. In patients with IPF, the incidence is up to 80%, and symptoms may persist despite the use of proton pump inhibitors.[25] Sleep apnea syndrome occurs in more than half of the patients. Predictors are a high body mass index (BMI) and deteriorating lung function.[24] Depression, diagnosed in approx. 25% of patients with ILDs, especially in the group with severe dyspnea, sleep disorders, markedly reduced FVC and many associated conditions, is related to a progressive reduction in physical activity.[29] Depression is an independent risk factor, and regular screening for its occurrence is recommended.[30]

Pulmonary diseases accompanying ILDs

Each pulmonary disease accompanying ILDs results in an additive effect, causing greater lung function abnormalities and increased mortality compared to each disease separately. A classic example is the coexistence of emphysema and ILDs. In 2005, Cottin et al. described a syndrome of coexistence of upper lobe emphysema and fibrosis of the lower lung lobes.[31] The incidence of emphysema in ILD cases is estimated at 10%, and primarily concerns smoking males, who are more likely to develop lung cancer and PAH. The frequency of home oxygen therapy is increased in this group. An increase in the incidence of lung cancer is observed in patients with IPF, systemic sclerosis, dermatomyositis, ascites, and asbestosis.[24]

The currently available literature presents 2 different opinions on responses to PR. Some authors estimate that greater improvement is observed in patients whose baseline FVC is higher and whose desaturation after exercise is lower. These authors conclude that it is crucial to introduce PR as early as possible in this group of patients.[29] Other authors observe that lower 6MWT baseline values result in the greatest increase after PR, suggesting that even in very advanced disease respiratory rehabilitation makes sense.[32] It seems that both opinions are correct, proving that improvements in ILD patients can be achieved in a variety of diseases with a broad spectrum of progression.[33]

The improvement in ILDs patients after PR persists for up to 6 months, although detailed evaluations are affected by the high percentage of patients who do not complete rehabilitation programs, indicated as 28% by Ryerson et al.[34] In a group of 36 patients with ILDs of varying etiology, 16 underwent a 6-month PR program, comprising a total of 60 sessions, and control tests were carried out 1 year after the conclusion. It turned out that improvement in lung function and exercise tolerance are visible even 6 months after finishing a program of this kind. Significant differences between the PR group and the control group were observed after a year, evaluating the 6MWT, muscle strength and maximal workload on a cycle ergometer.[35] In a group of 48 IPF patients qualified for lung transplantation, 12 weeks of PR were conducted. The patients were characterized by severe lung function impairment (average FVC was 49% ±13% of the predicted value and DLCO was 46% ±17%). The improvement was assessed on the basis of the 6MWT and the SF-36 questionnaire. Out of this group, 31 participants successfully completed the PR, and the rest of the respondents did not show any significant differences in the parameters assessed before the start of PR.[36]

In a meta-analysis of 9 PR programs for ILD patients, 8 of the programs were on an outpatient basis. The average duration was 10 weeks, with 2 sessions per week. Pulmonary rehabilitation complications are rare, mainly involving exercise-induced hypoxemia, ILD-specific symptoms and arrhythmias. Most authors recommend interrupting exercise when O_2 saturation drops below 80%. Continuing the exercise in such patients requires oxygen supplementation to achieve saturation above 85% and even up as high as 90%.[37] Oxygen supplementation during PR in patients with ILDs improves exercise tolerance.[38]

General rules of qualification for pulmonary rehabilitation in ILDs

Patients with a broad spectrum of ILDs are qualified for PR, most commonly with IPF, sarcoidosis and post-inflammatory pulmonary fibrosis. The qualification procedure should include:

1. a detailed interview;
2. a physical examination in which attention should be paid to the presence of musculoskeletal disorders that may significantly impair the patient's physical activity;
3. cardiac diagnostics, including an evaluation of ischemic changes, arrhythmias and cardiac conduction (especially in patients with sarcoidosis) as well as pulmonary pressure;
4. cardiac safety parameters, including a pulse rate at rest <120 bpm, right ventricular systolic blood pressure (RVSBP) <40 mm Hg, ejection fraction ≥40%, and no recent ischemic lesions visible on electrocardiography (ECG);
5. elimination of harmful environmental factors like smoking and exposure to allergens;
6. a diagnosis of accompanying diseases;
7. an evaluation of peripheral muscle strength;
8. the ability to live independently.

Pulmonary rehabilitation should be introduced as early as possible, especially for IPF patients. The basic safety parameter is a stable course of the disease. In CTD-ILDs (rheumatoid arthritis, systemic scleroderma), pain and stiffness of the joints can be a problem.

Contraindications to PR include:

1. an unstable course of the disease;
2. fainting after effort during an examination;
3. symptoms of right ventricular cardiovascular failure;
4. an uncontrolled course of accompanying diseases; and
5. any disease that prevents exercise training.[20]

Despite many uncertainties, the current state of knowledge allows PR to be included in standard care in ILD patients in 6–12-month intervals.[38] Due to the different etiology and prognoses of ILDs, the effects of PR will largely depend on the diagnosis. Especially in diseases with an acute onset, it is advisable to achieve remission or stabilize the disease before starting PR. In IPF, due to the rapid progression of the disease, early qualification for PR is recommended. The positive effects of PR can last up to 6 months.[32,39]

Numerous parameters are used to evaluate the effectiveness of PR in ILDs:

1. changes in the distance in 6MWT;
2. lowest O_2 saturation value in the 6MWT test (nadir SpO_2 less exercise-induced oxyhemoglobin desaturation);
3. resting RSVBP; and
4. scores on specific lung disease questionnaires (SGRQ, CRDQ and MRC-BDI).

The 6MWT in patients with IPF is highly repeatable at short intervals (1–2 weeks) and highly correlated (r = 0.78) with maximal oxygen uptake in the ergospirometry exercise test. The minimum significant difference (MCID) in the 6MWT has been determined as 28 m.[15] In another study, based on a study population 822 patients with IPF the estimated MCID was 24–45 m.[16] The total distance in the 6MWT provides an easy evaluation of physical capacity. The 6MWT score increases with the reduction of fatigue observed after aerobic training.[40] A gradual decline in the 6MWT is associated with increased mortality in ILD patients, especially those with IPF.[41] A meta-analysis of studies performed on small groups of patients (43 in total who underwent PR and 42 controls) showed that the patients with IPF demonstrated less improvement after PR than the patients with other ILDs. In the IPF group, an average increase in the 6MWT immediately after PR was 26.55 m; in the remaining patients with ILDs, it was 38.61 m. This may be related to the initial severity and faster course of the disease in IPF patients.[41] In a study conducted on a group of 32 patients with IPF, 15 were subjected to a 12-week exercise-based PR program and showed significant improvement in exercise tolerance, functional capacity and FVC. To evaluate exercise tolerance, the cardiopulmonary exercise test was used; after 12 weeks of PR, there was a significant increase in VO_2max. This is an interesting observation, because in patients with other lung diseases, e.g., COPD, there is no improvement in VO_2max and FVC after PR. Considering that the average survival time of IPF patients is 3–5 years from diagnosis, improvement in these parameters in a group of IPF patients an average of 2–3 years following their diagnosis shows that even in such a rapidly progressing disease, PR can cause measurable improvement.[42] Quality of life questionnaires and nadir SpO_2 are simple and credible ways to evaluate the patients' response to PR.[32]

The effectiveness of PR in patients with ILD is assessed differently in different publications. Differences are caused by:

1. different patient groups (usually there are better results in ILD groups with mixed etiology);
2. different patient conditions (in patients with IPF, short-term study results are less affected by the rapid disease progression);
3. different PR programs, lasting 12–24 weeks, with varied intensity (usually 2–3 times a week); and
4. varied parameters assessing the effectiveness of PR: exercise tolerance, functional capacity, pulmonary function tests, muscle strength, and health-related quality of life.

Conclusions

Pulmonary rehabilitation is an important part of comprehensive care for patients with ILDs. The principles of rehabilitation in this group of patients do not differ from the standards of PR for patients with COPD; the major

differences are due to poorer exercise tolerance and faster development of respiratory failure in patients with ILDs. Exercise-induced desaturation is frequently observed during PR. This is the main source of complications in patients with ILDs, and it occurs more frequently than in COPD patients. Pulmonary rehabilitation centers for ILD patients should be equipped with an oxygen source, a resuscitation kit, a defibrillator, and experienced staff. Early qualification and stabilization of the progression of ILDs and any accompanying diseases can reduce the risk of complications and achieve visible improvement in the patient's health status.

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Neuroendocrine tumors of the gastrointestinal tract and pancreas: Is it also a challenge for pediatricians?

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Abstract

Neuroendocrine tumors (NET) of the gastrointestinal tract and pancreas are extremely rare in the pediatric population and limited data is available. In most cases, NET of the gastrointestinal tract in children are located in the appendix. Pancreatic NET are a small but partially distinct group of the gastrointestinal neuroendocrine neoplasms. The most common in this group are insulinomas; however, in some research, the gastrinoma type neoplasms are perceived to be most common in children. This study reviews the typical clinical presentation, appropriate diagnostics, staging, and treatment of these uncommon neoplasms. It is important to know the epidemiology and symptomatology in this age group despite the fact that the majority of physicians treating the youngest patients will never have to deal with it. This will facilitate an early diagnosis in case of symptoms that may suggest neuroendocrine cancer. It appears necessary to create harmonized recommendations regarding the diagnosis, treatment and post-treatment follow-up for pediatric patients.

Key words: neuroendocrine tumors, pediatric, gastroenterology

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Introduction

Neuroendocrine neoplasms of the gastrointestinal tract and pancreas are a rare, heterogeneous group of neoplasms developing from the neuroendocrine system cells. Recent studies have shown that they derive from the local, multipotential progenitor cells that differentiate into neuroendocrine phenotype cells, rather than, as suspected before, from the migrating nerve crest cells.[1,2] This group includes tumors that produce hormones or biogenic amines, as well as the ones not producing such substances or not producing them in sufficient quantities to demonstrate clinical signs; such tumors are referred to as hormonally inactive. Neuroendocrine tumors (NET) are a major challenge for pediatricians, as they are very rare in children and data on symptomatology and clinical course in the pediatric population is limited.

Classification

Several different classifications have been proposed since this group of tumors was described about 100 years ago. Currently, the World Health Organization (WHO) guideline-based division, introduced in 2017, is being applied and it takes into account the histological tumor grade (G trait), pathomorphological (tumor-nodule-metastasis (TNM) system) and clinical staging. The most important parameter of a tumor is its histological maturity, evaluated using the proliferative index Ki-67 and its mitotic count. This has enabled the creation of 2 categories: the well-differentiated tumors with proliferative index Ki-67 < 20% and the poorly differentiated tumors with proliferative index Ki-67 > 20%. In 2017, the latter category included a subcategory of large and small cell neuroendocrine carcinomas (NEC) characterized by an exceptionally aggressive course and a subcategory of the well-differentiated neoplasms with Ki-67 proliferative index above 20% (NET G3) (Table 1).[3] Further treatment is determined by the category the tumor is assigned to.

Epidemiology

The incidence of NET in adults is estimated at about 5 cases per 100,000 people.[4] In children, the exact data is not known. Several studies assessing their incidence in children and young adults are available and they present values ranging from 1.14 per 1,000,000[5] to 2.8 per 1,000,000 people.[6] Neuroendocrine neoplasms of the gastrointestinal tract and the pancreas are the most common among all the NET in children and take the top position among all gastrointestinal neoplasms in pediatric patients.[7,8]

In adults, NET develop most frequently in the rectum or small intestine,[4] whilst in children they are most commonly located in the appendix. According to various studies, the average age for disease onset is 12–15 years.[9–12] The NET are more common in girls.

Pancreatic NET are a small but partially distinct group of gastrointestinal neuroendocrine neoplasms. The literature provides a very limited amount of data concerning their prevalence in the pediatric population. One study, which analyzed the cases of insulinoma within 60 years, demonstrated that pediatric patients (over 10 years of age) accounted for 4.9% of all cases. No gender predilection has been shown.[13] Insulinoma-type tumors are believed to be the most common in children, whilst in some research, the gastrinoma-type tumors are perceived to be most common in this group. A higher risk of pancreatic NET is observed in genetic syndromes such as multiple endocrine neoplasia type 1 (MEN1), von Hippel–Lindau syndrome, neurofibromatosis type 1 (NF1), and tuberous sclerosis.[14] A brief description of these syndromes has been presented in Table 2. One study showed that mutations associated with the MEN1 syndrome are much more common in children with insulinoma than in adults.[15]

VIPoma (VIP – vasoactive intestinal peptide), somatostatinoma and glucagonoma are extremely rare neuroendocrine neoplasms, which are practically not observed in children.

Symptomatology

In most cases, the diagnosis of NET of the gastrointestinal tract, due to its location in the appendix, is made accidentally during a histopathological examination, after an appendectomy was performed for another reason. Most frequently, they are small (<2 cm), hormonally inactive tumors without metastatic changes, with a very good prognosis and practically 100% survival.[16] The young age of the disease development may be associated with a larger size of tumor and a higher risk of metastases.

Table 1. Classification of NET staging according to WHO of 2017

NET G1	NET G2	NET G3	NEC
Proliferative index Ki-67 < 20%		proliferative index Ki-67 > 20%; typically 21–55%	proliferative index Ki-67 > 20%; typically >55%
Well-differentiated			poorly differentiated; divided into large- and small-cell carcinomas
Of low malignancy	of medium malignancy	less aggressive than NEC	extremely aggressive

NET – neuroendocrine tumor; NEC – neuroendocrine carcinoma.

Table 2. Hereditary syndromes associated with gastrointestinal and pancreatobiliary tract neuroendocrine tumors

Parameter	MEN1	Von Hippel–Lindau syndrome	NF1	Tuberous sclerosis
Mutation site	11q13	3p26-25	17q11.2	9q32
Inheritance patterns	autosomal dominant inheritance	autosomal dominant inheritance	autosomal dominant inheritance	autosomal dominant inheritance
Clinical characteristics	primary hyperparathyroidism; neuroendocrine neoplasms of pancreas, digestive tract, bronchi and thymus; pituitary tumors	increased risk of developing various neoplasms includes: cerebellar and spinal cord hemangiomas, retinal hemangiomas, clear cell carcinoma of kidneys, pheochromocytoma of adrenal glands, neuroendocrine tumors. They are bilateral and multifocal neoplasms, which develop at a young age	skin symptoms in the form of 'coffee-milk' stains, freckled spots in the armpit and groin areas, subcutaneous nodules with neuroma or neurofibroma interweaving pattern; furthermore, increased risk of tumors such as gliomas of the optic nerve, meningiomas of the brain and spinal cord, astrocytomas, rhabdomyosarcomas, pheochromocytoma and Wilms tumors	skin symptoms in the form of colorless nevi on limbs and trunk (may be present from birth), the Pringle syndrome demonstrated by red or pink nodules (angiofibroma) appearing on the face the age of 3, brown-yellow and convex fibroma of the forehead, subungual and periungual fibromas and scarring of the skin on the dorsal part of the body; additionally, the prevalence of the non-malignant tumors in various organs

MEN1 – multiple endocrine neoplasia type 1; NF1 – neurofibromatosis type 1.

In a small proportion of patients, the symptoms may be identical to those of appendicitis (most sources indicate acute abdominal pain; rare chronic abdominal pain in the right abdominal quadrant has also been reported[12]). Cases of carcinoid syndrome in the course of NET located in the appendix have been described in the adult population. This syndrome develops when metastases to the liver are observed. Then, the substances secreted by the neuroendocrine neoplasms are no longer inactivated by the liver and are the cause of, i.a., redness of the face and neck, tachycardia, dizziness, excessive sweating, diarrhea and, infrequently, bronchospasm. However, no such symptoms have been observed in studies among children with a diagnosis of NET of the appendix.[17,18]

Pancreatic NET are more frequently hormonally active than the gastrointestinal neuroendocrine tumor type. Hypoglycemia is the dominant symptom of insulinoma, a tumor which is derived from β-cells secreting insulin. The classic Whipple triad may be observed: symptoms occur during starvation and are accompanied by hypoglycemia; they disappear when carbohydrates are administered. In children, hypoglycemia usually manifests itself as behavioral disorders, convulsions or coma. These are non-characteristic ailments and may be observed in the course of many other diseases. Based on the available reports, the youngest age at diagnosis for pediatric insulinoma appears to be 3–4 years, thus this tumor is unlikely to be a cause of hypoglycemia in the youngest age group (<2 years of age). In most cases, insulinoma is small in size, i.e., about 2 cm, and is located mainly in the body and tail of the pancreas; approx. 1% of lesions are extrapancreatic. Ninety percent of all insulinomas are benign tumors.[19]

Gastrinoma is derived from G-cells that secrete gastrin. Typical symptoms for this NET include frequent, recurrent gastrointestinal ulcers manifested by chronic abdominal pain as well as symptoms suggesting reflux disease; less common are diarrhea and weight loss. Another symptom that may also be observed is anemia caused by abnormal iron absorption. All the aforementioned ailments are non-specific, which results in a diagnosis most frequently made with considerable delay, even up to 4–6 years after the occurrence of the first symptoms.[20] Approximately 30% of tumors are located outside the pancreas: mainly in the so-called gastrinoma triangle including the duodenum, peri-pancreatic soft tissues and regional lymph nodes. Gastrinoma usually reaches a diameter of about 4 cm and, unlike insulinoma, only 40% of its tumors contain benign features. Multiple tumors and metastases may occur much more frequently even at very small primary focus sizes. Gastrinoma, similarly to the NET of pancreas that are hormonally inactive, shows a predilection for the pancreatic head.

VIPoma (derived from D1 cells of pancreas that secrete a vasoactive intestinal peptide) is most frequently manifested by diarrhea, hypocalcemia, dehydration, and acidosis. Somatostatinoma (derived from the D cells) causes cholelithiasis, diabetes and steatorrhea. Symptoms characteristic for glucagonoma (made up of the A-cells) include necrolytic erythema, glucose intolerance and weight loss.

As it is written in the introduction there are NETs which secrete hormones or hormonally inactive. Hormonally inactive pancreatic NETs are detected much later and in more advanced stage. This results from the lack of secretion of active substances giving clinical symptoms; thus, the neoplasm remains asymptomatic for a long time. The diagnosis is usually made when the tumor reaches a size that causes compression of adjacent structures. The symptoms most commonly include abdominal pain, weight loss, lack of appetite, and vomiting; less common are hepatitis or palpable tumor in the abdominal cavity.[21]

Diagnostics

Determining the level of chromogranin A (Cg A) in the blood[22,23] is of essential significance in the laboratory diagnostics of NET. It is a sensitive but not a very specific marker. False positive results of the Cg A assay are observed when taking proton pump inhibitors (PPIs) in atrophic gastritis or renal failure, and after a meal or after physical activity which took place 2–4 h prior to blood sampling. The increased Cg A levels can also be caused by other neoplasms (small-cell lung cancer, medullary thyroid cancer, pheochromocytoma, liver cancer, or adenocarcinoma of the pancreas). The highest Cg A values were observed in NET of the small intestine, gastrinoma and glucagon, as well as in patients with carcinoid syndrome. For insulinoma, Cg A levels were often within normal limits, so the possibility of determining the level of chromogranin B should be considered for the diagnosis of insulinoma. A normal level of chromogranin A does not exclude the diagnosis of NET. There are several methods of determining Cg A, so it is important for the subsequent assessment of the response to treatment that determination of Cg A is performed using the same method.[22]

Following the guidelines of the Polish Society of Endocrinology, the gold standard of diagnostic management for insulinoma is a 72-hour hunger test (some sources give a sufficient time of 48 h, which may be helpful in diagnosing the youngest patients). The test must be carried out in a hospital environment. Initially, the blood glucose level is determined in series, until it reaches a value ≤2.2 mmol/L; then, the C-peptide, pro-insulin and insulin levels in the blood are determined. The diagnosis of insulinoma is based on the following criteria: documented glycemia ≤2.2 mmol/L (≤40 mg/dL) with concomitant inadequate insulin concentration ≥6 mU/L (≥36 pmol/L), C-peptide concentration ≥200 pmol/L, and pro-insulin concentration ≥5 pmol/L.[22,23]

If gastrinoma is suspected, the laboratory diagnosis includes: determination of the fasting serum gastrin concentration and evaluation of the gastrin concentration after being stimulated with secretin (2 J/kg body weight intravenously (i.v.)) or calcium gluconate in doubtful cases. The increased blood gastrin level, over 10 times higher than normal at gastric pH < 2, is typical, but does not enable a diagnosis because other causes of hypergastrinemia may also be accounted for, i.a., the use of PPIs, *Heliobacter pylori* infection, atrophic gastritis, pyloric stenosis, or renal failure; pH > 3 is very likely to exclude the existence of gastrinoma. The secretin stimulation test is performed on an empty stomach; secretin is administered i.v., followed by the determination of the baseline level (15 min and 1 min before secretin is administered) and of the gastrin levels in series within 30 min (2, 5, 10, 15, 20, and 30 min after secretin has been administered). Diagnosis is made when a value of 120 pg/mL above the baseline is obtained at any measuring point.[22,23]

It is advisable to carry out the appropriate test in patients under 20 years of age in connection with the significantly higher prevalence of MEN1 among people of that age, regarding a diagnosis of insulinoma and gastrinoma. Monitoring of such patients with endoscopic ultrasound (EUS) should also be considered, since it facilitates early detection of small pancreatic tumors, particularly the hormonally inactive NET of the pancreas,[23] which do not give early symptoms. This is followed by diagnostic imaging, which frequently includes not only anatomical but also functional imaging. The literature provides a limited amount of information on the imaging of neuroendocrine neoplasms in children. Most of the recommendations follow the practices accepted for adult patients. Computed tomography (CT) is highly sensitive in detecting both the primary focus and potential metastases.[24] However, it is associated with exposure of the youngest patients to radiation, so the test protocol should be selected in such manner to minimize the radiation to the lowest possible dose while maintaining the diagnostic values of the test. Enteroclysis or enterography, which increase the sensitivity of CT examination to 100%, are performed to provide a more accurate imaging of the small intestine.[25] Magnetic resonance imaging is characterized by higher sensitivity in detecting liver metastases[26] than CT, as well as by a very good detection of pancreatic tumors and lymph node metastasis. Endoscopic ultrasound is a good method of imaging pancreatic tumors located mainly in the head of the pancreas and duodenum.[27] Gastroscopy should be the first imaging examination in patients with a suspected cancer located in the upper gastrointestinal tract. Tissue samples may be collected when both the EUS and gastroscopy are being performed.

Functional imaging consists in the administration of somatostatin analogues labeled with radioisotope (somatostatin receptor imaging – SRI), showing affinity to glycoprotein receptors which are found in most neuroendocrine neoplasms. Currently, the most commonly used techniques include labeling with technetium (scintigraphy) or with positron markers (^{68}Ga; positron emission tomography (PET)) to locate the primary focus and metastases. Isotope diagnostics, using the radioactive tracer – fludeoxyglucose labeled fludeoxyglucose ^{18}(FDG), is used to evaluate the degree of NET malignancy. This examination produces negative results in a large number of patients with NET, but the accumulation of ^{18}FDG in neoplastic lesions indicates a high degree of cancer malignancy, which enables selection of an appropriate treatment line and an evaluation of prognosis. Location of the primary focus and the evaluation of the disease severity are the indications for SRI; it may also be used to monitor treatment.

Selection of the type of imaging examination should depend on the available technologies, applied research protocols and the staff experience in a given unit. Histopathological diagnosis of the gastrointestinal and pancreatic NET includes: histological type assessment according

to the WHO classification of 2017,[3] immune-histochemical evaluation of the expression of CgA and synaptophysin neuroendocrine markers, the proliferative activity of Ki-67/MIB1, and the histopathological level evaluation according to European Neuroendocrine Tumor Society (ENETS) and American Joint Committee on Cancer (AJCC) of 2017, as well as an evaluation of surgical margins.

Management

Unlike adults, in whom the treatment of NET located in the appendix depends on the size of the tumor, local infiltration and lymph node metastasis (patients with tumor >2 cm, infiltration to the adjacent structures or the lymph node metastasis are qualified for right hemicolectomy), it seems that in children, appendectomy is a sufficient treatment, regardless of the size of the tumor, its location in the appendix, lymph node metastasis, or intestine mesentery.[28,29] However, some sources recommend an approach analogous to the recommendations administered for adults.[30]

Treatment of NET located elsewhere mainly involves surgical management. The aim is to achieve a complete resection of the tumor, which guarantees full recovery of the patient. In children, NET are usually in the form of a small tumor, well-differentiated and non-infiltrating to adjacent structures, which usually allows for complete resection. No additional treatment of hormonally inactive NET is required in most cases.

Additional preoperative medication management is required in patients with insulinoma. An i.v. infusion of glucose is used, which is usually insufficient to maintain a normal level of glycemia, and a dose of 5–10 mg/kg/day of diazoxide is administered.[31]

Preoperative medication management is also required in patients with gastrinoma to inhibit gastric hypersecretion and avoid severe complications. Proton pump inhibitors used in high doses are the first-line drugs. The limited amount of data provided by the literature indicates that the metabolism of PPIs may be reduced in newborns, whereas in children between 1 and 9 years of age, it may be increased, which is worth noting when determining the dosage of these drugs. The aim of the treatment is to achieve a basal acid output (BAO) of less than 10 mEq/h.[32] The appropriate BAO in healthy people is 5 mEq/h.

Post-treatment control

Since NET occur in the pediatric population very rarely, there are no uniform recommendations for the post-treatment follow-up of NET in the youngest patients. Recommendations of the Polish Society of Endocrinology, created for adults, are justified to be applied in this respect:

– patients with benign, well-differentiated NET < 1 cm in diameter, with a total tumor resection (R0), do not require further monitoring in most cases;

– NET patients with histological type G1 should be monitored every 6–12 months;

– NET patients with histological type G2 should be monitored every 3–6 months;

– NET patients with histological type G3 should be monitored every 3 months.

Post-treatment monitoring includes: CgA level, 5-hydroxyindoleacetic acid (5-HIAA) level (in selected cases), three-phase CT, and somatostatin-receptor-based imaging.

It is also very important to have a genetic consultation for each newly diagnosed NET in a child. The aim is both to diagnose rare genetic syndromes that predispose to the development of NET, and thus increased monitoring to enable early detection of further cancers, and to provide genetic counseling for the whole family.

Summary

It is important to know the epidemiology and symptomatology of neuroendocrine neoplasms in this age group despite the fact that the majority of physicians dealing with the youngest patients will never encounter a case of NET in a child or will only encounter them a few times during their entire professional career. This will facilitate an early diagnosis in case of symptoms that may suggest neuroendocrine cancer. It appears necessary to create harmonized recommendations regarding the diagnosis, treatment and post-treatment follow-up for pediatric patients.

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